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PREFACE

In this edition volume 20 no.2 June 2015, we publish Jurnal Ilmu Ternak dan Veteriner in English. We will henceforth always publish this journal in English. This is done because this journal can be accessed from around the world, then an English version is needed in order to get a wider audience.

The articles published in this edition are: "Multivariate analysis of morphometric traits of three different indigenous cattle Populations from North East states of India"; “Isolation and identification of indigenous lactic acid bacteria from North Sumatra river buffalo milk"; "Effectivity of probiotic, micro mineral enriched yeast and their combination with *Azadirachta indica* leaves containing tannin on fermentability and digestibility of *Pennisetum* hybrid"; "Productivity of *Calliandra calothyrsus*, *Indigofera zollingeriana*, and *Gliricidia sepium* on acid soil in the greenhouse"; "Molecular analysis of the hemagglutinin gene of avian Influenza viruses isolated in 2012-2013"; "Reliability of the DIVA test based on M2E exceed Reviews those on HA2 peptide or peptides NSI"; "Newcastle Disease Virus infection study on duck and chicken in Subang district"; and "Curcumin effect on Bleomuchin-induced pulmonary fibrosis in *Mus musculus*"

Hopefully these articles would be useful for their readers and farmers the users of technological innovation needed for the development of the world of farming.

Bogor, June 2015

Chief Editor

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Multivariate Analysis of Morphometric Traits of Three Different Indigenous Cattle Populations from North East States of India

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ABSTRAK

Pundir RK, Singh PK, Sadana DK. 2015. Analisis multivariate sifat-sifat morfometrik pada tiga populasi sapi asli bagian Timur Laut India yang berbeda. JITV 20(2): 79-86. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1162

Penelitian ini dilakukan untuk membedakan 3 populasi sapi bagian Timur Laut India yaitu Tripura, Mizoram, dan Maniour berdasarkan sifat-sifat morfometrik menggunakan analisis pembeda canonical untuk melihat apakah mereka sama atau berbeda. Data terdiri dari 8 sifat morfometrik yang berbeda dari 383 sapi asli Tripura (136), Mizoram (71) dan Manipur (176). Sifat morfometrik berupa panjang tubuh, tinggi bagian punggung tertinggi, lingkar jantung, lingkar tembolok, lebar wajah, panjang telinga, panjang tanduk, dan panjang ekor tanpa sendi. Semua sifat morfometrik yang diamati memiliki perbedaan yang signifikan untuk semua populasi kecuali pada panjang tanduk. Nilai semua sifat pada sapi Tripura lebih rendah dibandngkan sapi Mizoram dan Manipur. Analisis pembeda bertahap menunjukkan bahwa tinggi bagian punggung tertinggi, panjang badan, panjang telinga, panjang ekor tanpa sendi, lingkat tembolok, and panjang wajah merupakan sifat yang paling berbeda diantara ketiga populasi sapi tersebut. Jarak Mahalanobis berpasangan antara populasi sapi Tripura dan Mizoram, Tripura dan Manipur, serta Mizoram dan Manipur adalah 9,72578, 5,72089 dan 4,65239 berturut-turut dan signifikan. Dendogram menunjukkan bahwa terdapat 2 kelompok, kelompok 1 terdiri dari sapi Manipur dan Mizoram serta kelpompok 2 adalah sapi Tripura yang di[isahkan secara jelas dari kelompok 1. Penempatan individu dari populasi yang berbeda dengan pengelompokkan validasi silang mengungkapkan bahwa 84,13% sapi Tripura, 82,09% sapi Mizoram, dan 79,87% sapi Manipur ditempatkan dengan benar di dalam populasi masing-masing. Berdasarkan pengamatan ini, kita tidak dapat menyimpulkan bahwa mereka adalah tiga bangsa yang berbeda. Tetapi, informasi saat ini, pada tiga populasi sapi tersebut dapat di dimanfaatkan dalam perancangan strategi-strategi yang tepat untuk managemen dan pelestarian sapi-sapi tersebut.

Kata Kunci: Sapi Asli, Sifat Morfometrik, Analisis Multivariate, Analisis Kelompok, Analisis Pembeda Kanonikal

ABSTRACT

Pundir RK, Singh PK, Sadana DK. 2015. Multivariate analysis of morphometric traits of three different indigenous cattle populations from North East states of India. JITV 20(2): 79-86. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1162

In the present study an attempt has been made to differentiate three cattle populations of North East states of India i.e. Tripura, Mizoram and Manipur based on morphometric traits, using canonical discriminant analysis to see whether they are similar or distinct. Data consisted of eight different morphometric traits of 383 indigenous cows from Tripura (136), Mizoram (71) and Manipur (176). Morphometric traits included body length, height at withers, heart girth, paunch girth, face length, ear length, horn length and tail length without switch. All the morphometric traits under study differ significantly in these populations except horn length. All the traits, values were lower in Tripura cows than that of Mizoram and Manipur cows. The stepwise discriminant analysis showed that height at withers, body length, ear length, tail length without switch, paunch girth and face length were the most discriminating traits in these three cattle populations. The pair wise Mahalanobis distances between Tripura and Mizoram, Tripura and Manipur and Mizoram and Manipur were 9.72578, 5.72089 and 4.65239, respectively, and significant. The dendogram showed that there are two clusters; cluster one includes Manipur and Mizoram cows and cluster two Tripura cows those are clearly separated from cluster one. The Individual assignment of different cattle populations by the cross-validation classification revealed 84.13% of Tripura cows, 82.09% of Mizoram cows and 79.87% Manipur cows were assigned correctly into their respective population. Based on the present study we cannot conclude that they are three different distinct breeds. However, the present information on the three cattle populations could therefore be exploited in designing appropriate strategies for their management and conservation.

Key Words: Indigenous Cattle, Morphometric Traits, Multivariate Analysis, Cluster Analysis, Canonical Discriminant Analysis

INTRODUCTION

North East states of India comprises of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura states (Figure 1 and 2). India had 190.9 million cattle heads in the year 2012 including 151.17 million indigenous and 39.73 million exotic and crossbreds (LC 2012). All these states has around 6.9% of total cattle population of the country. The proportion of indigenous and exotic cattle as compared to whole country was 8.19% and 2.28% in these states (Table 1). In this region, there is only one registered cattle breed known as Siri (Sikkim). Rest of the cattle in this region is known as known as *Desi* (non described). There is a need to characterize these cattle populations available in these states and observed similarity/dissimilarity with existing populations using multivariate techniques. If such populations are found distinct/unique, then register them as a distinct breed and if not, search a breed/ population where they can be merged or mixed.

Previous efforts on the phenotypic characterization of breeds of livestock have been restricted to the use of analysis of variance, whereas the current trends in livestock classification involve the use of multivariate statistical tools (Traore et al. 2008; Yakubu & Akinyemi 2010; Peter et al. 2012; Aziz & Al-Hur 2013). Univariate statistical analysis analyzes each variable separately and do not explain how the populations under investigations differ when all measured morphological traits are considered simultaneously (Dossa et al. 2007). Multifactorial discriminant analyses have been found to be more suitable in assessing variation within a population and can discriminate different population types when all measured morphological traits are considered jointly. Discriminate function analysis can be used not only as a means to explain differences among populations, but also to predict group membership for sampling entities of unknown membership. Discriminate analysis has been used for differentiating populations utilizing various morphological measurements simultaneously (Herrera et al. 1996; Capote et al. 1998; Zaitoun et al. 2005; Dossa et al. 2007; Martins et al. 2009; Yakubu et al. 2010a; Yakubu et al. 2010b; Yakubu et al. 2010c; Peter et al. 2012; Aziz & Al-Hur 2013). In the present study an attempt will be made to differentiate between three cattle populations of North East states of India i.e. Tripura, Mizoram and Manipur based on morphological traits, using canonical discriminant analysis to see whether they are distinct or similar.

Table 1. Cattle population (in thousands) in north east states in India in the year 2012

State	Cattle population	Indigenous cattle	Indigenous female	Exotic cattle
Arunachal Pradesh	463.76	440.53	248.67	23.23
Assam	10307.60	9911.70	5695.29	395.90
Manipur	263.84	219.54	133.80	44.31
Meghalaya	896.00	860.75	513.61	35.25
Mizoram	34.57	23.28	14.75	11.30
Nagaland	234.97	106.02	64.46	128.95
Sikkim	140.47	13.95	8.90	126.52
Tripura	948.79	815.69	502.89	133.31
Total	13290.00 (6.96%)	12391.46 (8.19%)	7182.37 (8.05%)	898.77 (2.28%)
India	190904.00	151172.00	89223.00	39731.00

Source: 19th LC 2012 (http://dahd.nic.in/dahd/WriteReadData/Livestock.pdf)

Figure 1. North East zone of India
Source: http://www.mapsofindia.com/states/

Figure 2. North East zone of India
Source: http://www.mapsofindia.com/states/

MATERIALS AND METHODS

Collection of data and location of study

Data consisted of 8 different morphometric traits of 383 indigenous cows from Tripura (136 from West, South, Gomti and Dhalai districts), Mizoram (71 from Champhai and Kolasib districts) and Manipur states (176 from Imphal East, Imphal west and Churachandpur districts) of the union of India. These indigenous cattle in all the three states were not described earlier and so far known as nondescript/desi. All the measurements were recorded by the same recorder to avoid between recorder effects. All the traits were recorded from the left side of the cows. The circumference measurements were taken from a tape while the other measures were taken by a measuring stick. Cows were reared through the extensive management system and originated from different herds in different states.

Measured traits

The recorded morphometric traits were body length (the distance from the point of the shoulder joint to the point of the pin bone), height at withers (the distance from the highest point of withers to the ground), heart girth (the circumference of the chest just behind the elbow joint), paunch girth (the circumference at paunch region just anterior to the hip joint), ear length (distance from the point of attachment of ear to the tip of the ear) face length(distance from between the horn site to the lower lip), horn length (distance from part of horn attachment to the tip of the horn) and tail length without switch (measured from the root of tail droop to the tip of the tail excluding switch). Physical traits like coat colour, body shape, face, horns, udder and tail characters were also recorded.

Statistical analysis

Means, standard errors and coefficients of variation of the different morphometric traits were calculated using General linear model PROC GLM (SAS 2009) with state effect. The DUNCAN's multiple range test was performed by all the means of different morphometric traits to see whether states are differ significantly or not. Stepwise discriminate procedure (SAS 2009) was applied using PROC STEPDISC to determine which morphological traits have more discriminant power than others. The relative importance of the morphometric variables in discriminating between the cattle populations was assessed using the level of significance, partial R2 and F-statistic. The CANDISC (SAS 2009) procedure was used to perform univariate and multivariate one-way analysis that

calculated the Mahalanobis distances between the three cattle populations. Based on the Mahalanobis distance matrix dendogram was created using PROC CLUSTER (SAS 2009) with Average Linkage Method. The ability of these canonical functions to assign each individual animal to its respective population calculated as the percentage of correct assignment to each cattle population using the DISCRIM (SAS 2009) procedure by Nearest Neighbour Discriminant Analysis. The cross-validation approach was used for assignment of individual to their respective population in which one individual is removed from the original matrix and the discriminant analysis is then performed from the remaining observations and used to classify the omitted individual. It also providesan unbiased estimate of error. The proportion of individuals correctly re-allocated is taken as a measure of the morphological distinctness of the population.

RESULTS AND DISCUSSIONS

Tripura, Mizoram and Manipur states are adjoining and located in eastern part of the country. In these states temperature ranged from 10°C to 32ºC. Rice is major crop and no green fodder was grown for animals. Annual rainfall is high more than 2000 mm. Animals were reared mainly on extensive system of management i.e. grazing from morning to evening. Physical traits recorded on these three cattle populations did not reveal significant differences as majority of traits were over lapping. Analysis of physical traits (frequencies) in these cattle population showed that they are differing in proportion of different physical traits, but there was not a single physical trait which can differentiate them strictly. In general animals were small in size with the cylindrical type of body. Animals were well built and compact with strong legs. The coat colour varied in different colours i.e. brown, black and grey/white but brown colour predominates. Dewlap and hump were small. The head was small. Face was short and concave. Ears were small to moderate in length and horizontal in orientation. The neck was short in length and thin. Horns were small, black or gray in colour. Orientation was outward and then upward. Hoofs were black. Muzzles were brown and black. Udder was small, not well developed and milk veins were not prominent. Sizes of fore and rear udder were small. Teats were small 5-12 cm long. Penis sheath flap was short and tucked up with the body. The tail was longer up to the hock with black, brown and white switch. Temperament was docile in all the cases. Cows of these three cattle populations are presented in Figures 3-5.

Descriptive statistics of the morphological traits of three different indigenous cows from three different states are given in Table 2. All the traits under study differ significantly in these populations except horn length. All the traits, values were lower in Tripura cows than that of Mizoram and Manipur cows. Manipuri and Mizoram cows differ significantly in body length, ear length and tail length without switch.

The considerable variation in body dimensions of the three cattle populations might not be unconnected with individual population potential and peculiarities. The minimum and maximum variability was observed in horn length and ear length, respectively. The estimates of body length obtained in the present study were in agreement with the reports of Pundir et al. (2013) in Uttara cows, Pundir et al. (2012) in Pithoragarh cows and Pundir et al. (2009) in Bargur cows. However, higher estimates of body length were observed by Singh et al. (2012) in Pullikumam cows, Pundir et al. (2011) in Kankrej cows and Pundiret al. (2007) in Kenkatha cows.The estimates of height at wither, heart girth and paunch girth were comparablewith the reports of Pundir et al. (2012; 2013). Higher estimates of height at wither were reported by Singh et al. (2012), Pundir et al. (2007; 2011).

Figure 3. Indigenous cattle of Tripura

Figure 4. Indigenous cattle of Manipur

Figure 5. Indigenous cattle of Mizoram

Table 2. Descriptive statistics of different morphometric traits (cm) in indigenous cows of NEH states

State	Overall (383)		Tripura (136)		Mizoram (71)		Manipur (176)	
Trait	Mean+S.E.	C.V.	Mean+S.E.	C.V.	Mean+S.E.	C.V.	Mean+S.E.	C.V.
Body lenght	101.14+0.46**	8.75	98.09+0.54^{c}	6.43	109.03+1.39^{a}	10.77	100.32+0.59^{b}	7.88
Height at wither	101.80+0.45**	8.81	93.39+0.44^{b}	5.52	106.92+0.84^{a}	6.67	106.22+0.51^{a}	6.48
Heart girth	132.45+0.72**	10.72	122.05+1.09^{b}	10.46	139.52+1.59^{a}	9.67	137.69+0.78^{a}	7.53
Paunch girth	136.89+0.82**	11.67	125.41+1.08^{b}	10.13	146.64+1.80^{a}	10.41	142.12+0.98^{a}	9.10
Ear length	19.26+0.13**	13.70	19.47+0.24^{b}	14.38	18.02+0.33^{a}	15.88	19.59+0.17^{b}	11.58
Face length	36.73+0.15**	8.63	35.30+0.20^{b}	6.71	36.15+0.37^{a}	8.82	38.06+0.23^{c}	8.02
Tail length without switch	71.20+0.38**	10.57	68.63+0.51^{a}	8.88	68.54+1.07^{a}	13.23	74.31+0.50^{b}	8.96
Horn length	11.34+0.26	4.37	10.87+0.50	5.20	11.01+0.42	31.90	11.85+0.38	41.01

Table 3. Summary of step wise selection of different traits in indigenous cows of of NEH states

Variable Entered	Partial R-Square	F Value	Pr>F	Wilks' Lambda	Pr< Lambda	Average Squared Canonical Correlation	Pr> ASCC
Height at wither	0.5114	180.06	<0.0001	0.488	<0.0001	0.255	<0.0001
Body length	0.1315	25.96	<0.0001	0.423	<0.0001	0.320	<0.0001
Eal length	0.1814	37.90	<0.0001	0.347	<0.0001	0.390	<0.0001
Tail length without switch	0.1112	21.33	<0.0001	0.308	<0.0001	0.432	<0.0001
Paunch girth	0.0852	15.84	<0.0001	0.282	<0.0001	0.451	<0.0001
Face length	0.0770	14.14	<0.0001	0.260	<0.0001	0.477	<0.0001

Similar estimation of face length and tail length without switch were obtained by Pundir et al. (2007; 2012; 2013). Higher estimates of both the traits were observed by Singh et al. (2012), Pundir et al. (2009; 2011). Comparable estimates of horn length to the present study was reported by Pundir et al. (2013) However, Pundir et al. (2007; 2009; 2011; 2012) and Singh et al. (2012) obtained higher estimates of the same trait.

The stepwise discriminate analysis showed that height at wither, body length, ear length, tail length without switch, paunch girth and face were the most discriminating variables between these three cattle populations (Table 3). Their respective partial R2 were 0.5114, 0.1315, 0.1814, 0.1112, 0.0852 and 0.0770, respectively, with high significant values (P<0.0001). The corresponding F values for these traits were 180.6, 25.96, 37.90, 21.33, 15.84 and 14.14, respectively and highly significant.

These six morphological variables obtained in the present study are more important and informative, and could be used to assign the three cattle populations into distinct populations, thereby reducing the errors of selection in future breeding and selection programmes. Similar to the present study, Yakubu et al. (2010a) also reported height at wither and face length most discriminating traits in two distinct cattle breeds. In an attempt to distinguish between brown and gray Bengal goats, Mukeherjee et al. (1979) reported significant differences between both breeds due to body length and chest circumference.

Herrera et al. (1996) employed discriminate analysis on several body measurements such as, shin circumference, chest girth, chest depth, rump length and width, and shoulder height to differentiate among five Spanish goat breeds. Zaitoun et al. (2005) applied discriminant analysis on 20 metrical variables to discriminate among different goat genetic groups.

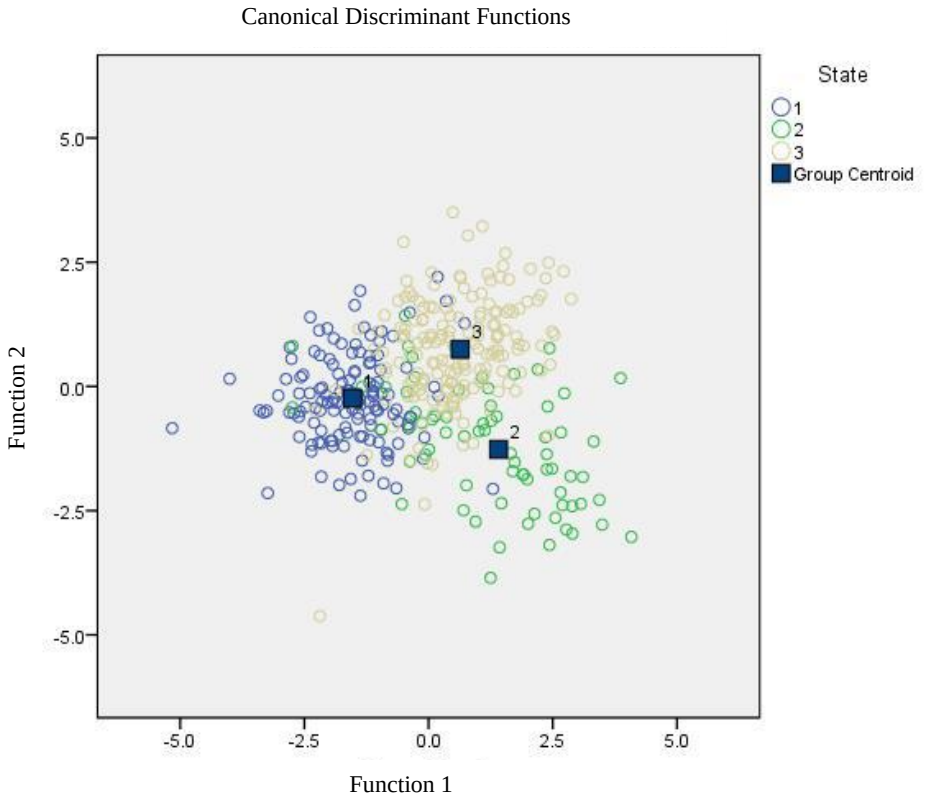

Figure 6. Canonical discriminiant functions of three indigenous cows populations (State 1 Tripura 2 Mizoram 3 Manipur)

In these studies, step-wise discriminant analysis was first applied to select the most important discriminator variables used for differentiation among breeds under study. The canonical discriminant function representation is shown in Figure 6 which revealed overlapping of these populations in the present study.The Mahalanobis distances between three cattle populations are given in Table 4. The pairwise distance Tripura and Mizoram, Tripura and Manipur and Mizoram and Manipur were 9.72578, 5.72089 and 4.65239, respectively, and highly significant (P<0.0001). Yakubu et al. (2010a) Observed Mahalanobis distance between the two cattle populations as 7.19 which was high and significant and indicated that that they belong to genetically different groups. Yakubu et al. (2010c) estimated Mahalanobis distance of 72.28 between West African Dwarf and Red Sokoto goats in Nigeria, indicating that there is considerable genetic variation between both breeds. Aziz & Al-Haur (2013) observed Mahalanobis distance of 0.55 between two lines of goat and between Ardi and each of Line1 and Line2 were 25.03 and 21.45, respectively.

The dendogram (Figure 7) based on the average linkage methodshowed that there are two clusters; cluster one includes Manipur and Mizoram cows and cluster two Tripura cows those are clearly separated from cluster one.

Table 4. Mahalanobis distances between three different populations of indigenous cows in North East States

State	Tripura	Mizoram	Manipur
Tripura	0	9.72578	5.72089
Mizoram	P<0.0001	0	4.65239
Manipur	P<0.0001	P<0.0001	0

In the lower triangular probability of significance is shown

The individual assignment to different cattle populations by the Cross-validation classification was given in table 5. The proportion of individuals correctly assigned to their respective population is considered as a measure of the morphological distinctness of the population.High values of error 0.158, 0.179 and 0.201 were observed for Tripura, Mizoram and Manipur cattle populations, respectively. The reason for this misclassification may be a high degree of intermingling these populations as they are from the adjoining states. The high morphological distances between the cattle populations coupled with high correct assignment to source populations is an indication that they belong to different populations. But there was no distinct physical trait which could differentiate these populations.

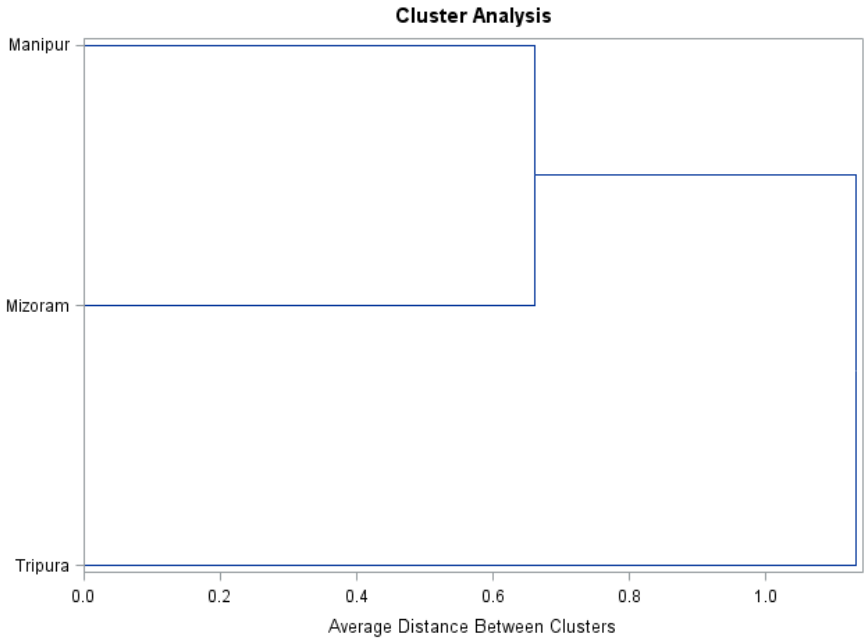

Figure 7. Dendogram showing similarity/distinctness in three indigenous cows populations

Table 5. Percent of individual cows classified in to different populations of north east states

Population	Tripura	Mizoram	Manipur	Total
Tripura	106	7	13	126
	(84.13)	(5.56)	(10.32)	(100)
Mizoram	4	55	8	67
	(5.97)	(82.09)	(11.94)	(100)
Manipur	13	18	123	154
	(8.44)	(11.69)	(79.87)	(100)
Error level	0.158	0.179	0.201	0.158
Priors	0.333	0.333	0.333	

Yakubu et al. (2010a) reported that 85.48% of Bunaji cattle and 96.55% of Sokoto Gudali classified into their source population assigned correctly by the Nearest Neighbour Discriminant Analysis. Aziz & Al-Haur (2013) observed 100% assignment of Ardi animals into their genetic group and percentages of animals assigned in Line1 and line2 were 86.10 and 42.55, respectively. The use of multivariate discriminant analyses therefore could be successfully used in morphometric differentiation. Similar reports on goats (Dossa et al. 2007; Yakubu et al. 2010a; Yakubu et al. 2010b; Yakubu et al. 2010c), sheep (Traore et al. 2008; Yakubu & Akinyemi 2010), cattle (Ndumu et al. 2008) and buffalo (Johari et al. 2009) respectively were observed.

CONCLUSION

In the present study correct assignment of individual animals to their respective population ranged from 80 to 84% but we could not get a physical /discontinuous trait which can distinct these populations may be due to intermingling. Canonical discriminiantanalysis also showed that these three indigenous cow populations were overlapping, so we cannot conclude that they are distinct breeds.

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Isolation and Identification of Indigenous Lactic Acid Bacteria from North Sumatra River Buffalo Milk

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ABSTRAK

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Susu kerbau merupakan salah satu sumber bakteri asam laktat (BAL) yang berpotensi sebagai kultur untuk starter maupun probiotik. Penelitian ini bertujuan untuk mengisolasi dan mengidentifikasi BAL asal susu kerbau sungai Sumatera Utara, serta menguji ketahanannya pada pH rendah. Bakteri asam laktat diisolasi dan ditumbuhkan pada medium *De Man Rogosa Sharpe Agar* (MRSA). Isolasi dilakukan sampai diperoleh kultur murni. Identifikasi BAL dengan pengujian karakteristik morfologi, fisiologi dan biokimia. Karakteristik morfologi dilakukan dengan pewarnaan Gram dan melihat bentuk sel. Karakteristik fisiologi dilakukan dengan uji ketahanan pada pH 4,5 dan suhu 45^{o}C. Karakteristik biokimia dilakukan dengan uji produksi CO_2, NH_3 dan dekstran. Pengujian sifat probiotik dilakukan dengan menguji ketahanan isolat BAL pada pH rendah. Pengujian dengan kit API tes CHL 50 untuk mengetahui jenis spesies dari sepuluh BAL terpilih. Hasil identifikasi diperoleh 41 isolat BAL, yang secara umum memiliki karakterisitik Gram positif, katalase negatif, berbentuk batang dan bulat. Pengujian ketahanan BAL pada pH rendah (pH 2) menunjukkan penurunan jumlah populasi sebanyak 1,24±0,68 log cfu/ml. Isolat terbaik dari pengujian ketahanan pada pH rendah adalah isolat L12, L16, L17, L19, L20, M10, P8, S3, S19 dan S20. Hasil pengujian dengan API test CHL 50 pada sepuluh isolat terpilih tersebut menunjukkan ada empat spesies BAL dari susu kerbau sungai Sumatera Utara, yaitu *Lactobacillus plantarum, L. brevis, L. pentosus* dan *Lactococus lactis*.

Kata Kunci: Susu Kerbau, BAL, Isolasi, Identifikasi

ABSTRACT

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Buffalo milk is a source of various lactic acid bacteria (LAB) which is potential as culture starter as well as the probiotic. This study was conducted to isolate and identify LAB from indigenous North Sumatra river buffalo milk. Lactic acid bacteria was isolated and grown in medium De Man Rogosa Sharpe Agar (MRSA). The isolation was conducted to obtain pure isolate. The identification of LAB was studied in terms of morphology, physiology, biochemistry and survival on low pH. Morphology tests were conducted by Gram staining and cell forming; physiology tests were conducted for growing viability at pH 4.5 and temperature at 45^{o}C; whereas biochemistry tests were conducted for CO_2, dextran and NH_3 productions. Determination of LAB species was conducted using Analytical Profile Index (API) test CHL 50. Results of identification showed that 41 isolates were identified as LAB with Gram-positive, catalase-negative, rod and round shaped characteristics. Resistance test done to low pH (pH 2) for the lactic acid bacteria showed decrease of bacteria viability up to1.24±0.68 log cfu/ml. The resistant isolates at low pH were L12, L16, L17, L19, L20, M10, P8, S3, S19 and S20. Identification with API test CHL 50 for 10 isolates showed that four isolates were identified as *Lactobacillus plantarum, L. brevis, L. pentosus* and *Lactococuslactis*.

Key Words: Buffalo Milk, LAB, Isolation, Identification

INTRODUCTION

Milk is one of food stuffs that naturally containing Lactic Acid Bacteria (LAB) which is generally used for culture starter forming various processed food products. The LAB originated from milk is also potentially developed into probiotic for functional food development. Probiotic was life bacteria, given as food and feed supplement which have beneficial effects for health of both human and animals by improving intestinal microflora balance (Roberfroid 2000). Study of LAB's potential as probiotic of indigenous materials are continuously done, especially to be used as functional food.

LAB isolation of various milk had been done, among others from raw cow milk (Abdullah & Osman 2010), goat milk (Tserovska et al. 2002; Setyawardani et al. 2013), Bima horse milk (Antara et al. 2009), wild horse milk (Sugitha et al. 2011), Sumbawa horse milk (Sujaya et al. 2008), sheep milk (Iranmanesh et al. 2012), camel milk (Ahmed et al. 2002; Khedid et al. 2009; Abbas & Mahasneh 2015), and breast milk (Nuraida et al. 2011). LAB isolation of fresh buffalo milk was done in India, Pakistan and Egypt both of fresh buffalo milk or produced buffalo milk products (Aziz et al. 2009; Tambekar et al. 2009; Singh & Sharma 2009; Patel & Patel 2012; Sharma et al. 2013; Shafakatullah & Chandra 2014; Kumar et al. 2014). In Indonesia, LAB isolation from the produced buffalo milk products had been done to produce a product of West Sumatran buffalo called "dadih" (Surono 2003; Sunaryanto & Marwoto 2013), whereas isolation and identification of LAB from fresh buffalo milk in Indonesia has not been much done.

North Sumatra river buffalo is one of genetic sources of Indonesian local animal which has not been explored. River buffalo milk production could reach 8 liter/day. Its quality was better compared to cow milk, where in the content of protein and fat was higher but its cholesterol content was lower (Damayanthi et al. 2014). Exploration of LAB from North Sumatra river buffalo and its utilization to produce the culture starter of probiotic product currently have not been much reported. This LAB exploration was first step to get probiotic bacteria candidate by testing several probiotic characteristics (Rizqiati et al. 2015); in further it was applied in mozzarella cheese into probiotic mozzarella cheese with North Sumatra river buffalo milk as raw material.

One condition for microorganism called as probiotic was its capability to survive in intestinal track condition such as endurance to low pH and bile salt (Nuraida et al. 2011). The first exposure of probiotic when entering the intestinal track was a gastric with pH around 2. Endurance test to low pH was needed to determine whether the LAB culture was survive in the gastric acid or not (Wildman & Medeiros 2000).

This study was aimed to isolate the LAB from North Sumatra river buffalo milk and identify it based on morphology, physiology, biochemical, and identify species of the LAB. Besides, this study was aimed to select LAB isolate which could survive in low pH (pH 2) which was one of probiotic characteristic tests. Isolation and identification results of LAB became first step of selection of LAB from North Sumatra river buffalo milk as a probiotic.

MATERIALS AND METHODS

Materials used in this study were 24 samples of river buffalo milk from 4 locations of buffalo farming in North Sumatra, namely: Lubuk Pakam, Medan, Patumbak, and Siborong-borong where from each location was taken 6 samples. The milk samples were brought in cold sterile tube in the cooling box to the Integrated Laboratory of Faculty of Animal Science of Bogor Agricultural University. Conducted tests were isolation and identification of LAB from the river buffalo milk. The isolation was done using media De Man Rogosa Sharpe (MRS). LAB identification consisted of characteristic of morphology, physiology, and biochemical. Characteristic tests of the probiotic were done by testing LAB resistance to low pH. LAB identification by API test CHL 50 was done to determine its species.

LAB isolation

LAB isolation technique referred to Khedid et al. (2009) which was modified. The buffalo milk samples (10 ml) was taken aseptically and inserted into 90 ml of sterile physiological NaCl solution (0.85%), and further it was done a gradual dilution into 10^5 dilutions. One ml diluted sample was distributed on MRS agar (MRSA) media containing of bromo cresol purple (BCP) 0.01% on petridish, and then the samples were incubated anaerobically for 24 hours at 37^{o}C. LAB colony seemed as colony which was surrounded by yellow zone, and further that colony was taken and scratched on MRSA media. That scratching was done continuously to obtain one pure colony.

Morphology characteristic to identify the LAB

Characterizing of cell morphology was aimed to see isolate form and Gram staining characteristic. Cell form was viewed by microscope and done after the Gram staining. Cell morphology expected was positive Gram with tube or oval cell (Iranmanesh et al. 2012).

Physiology characteristic to identify the LAB

Physiology characteristic test to identify the LAB consisted of resistance test to temperature and pH. Resistance test to temperature was done to select LAB which could still grow at 45^{o}C and 37^{o}C for 2-5 days as control. Resistance test to pH was aimed to select LAB isolate which could still grow at MRSB media with acid

environment with pH of 4.5 and in neutral condition (pH 7) as control, and further were incubated at 37ºC for 7 days. Growth was marked by turbidity in the media (Aziz et al. 2009).

Biochemical characteristic to identify the LAB

Biochemical characteristic test to identify the LAB consisted of catalase test, production test of CO_2, dextran, and NH_3. CO_2 production test was conducted to determine isolate capability to produce CO_2 from glucose, which showed whether the isolate was homofermentative or heterofermentative. Catalase test was done to determine the isolate capability to produce catalase enzyme. Catalase test was done using hydrogenperoxide (H_2O_2) 3%. Dextran production test was done to determine whether the isolate could produce dextran (mucus formed) which was generally produced by *Leuconostoc* genus. NH_3 production test was done to determine isolate which could produce ammoniac which was generally produced by *Streptococcus* genus (Harrigan 1998).

Probiotic characteristic testing of LAB resistance in low pH

LAB resistance test to low pH was done using PBS media by regulating the pH into 2 using HCL 0.1 N and incubated for 3 hours at 27ºC. LAB number was counted before and after the incubation. Cell number was counted by casting method using MRSA. Each test was done 3 times duple repetition (Lin et al. 2006).

LAB identification using kit API CHL 50

LAB identification using kit API CHL 50 (Biomerieux, France) was conducted to determine species of the LAB. The testing was done by 1 ose of LAB inserted into 10 ml MRSB media then incubated at 37ºC for 24 hours. LAB culture was centrifuged 9800 x g for 10 minutes. Separated pellet was inserted into API 50 CHL media by sterile pipette and homogenized. The culture inserted into 50 pits of kit API CHL 50 strips. All of the pits were closed by paraffin oil to give anaerobic environment and incubated at 37ºC for 24-48 hours. Testing parameter was color change from blue into yellow after incubation for 24-48 hours because of formation of acid which was detected by pH change. Observation result was processed by software Apiweb™ (Gawad et al. 2010).

RESULT AND DISCUSSION

LAB isolates

LAB isolation used MRSA media, added by BCP as bacteria indicator of acid former, where the isolate which produced acid, would form yellow zone. LAB colony appearance was in form of white oval surrounded by yellow zone. It was obtained 96 isolates from North Sumatra river buffalo milk from 4 locations, namely: Lubuk Pakam (L), Medan (M), Patumba (P), and Siborong-borong (S), where each location was obtained successfully 24 isolates. LAB colony grew in the MRSA media, which was added by BCP, would seem as colony surrounded by yellow zone (Surono 2004).

LAB identification

Morphology characteristic of LAB isolate was identified through Gram staining. It showed that from 96 pure isolates which was isolated successfully from river buffalo milk, 84 isolates were positive Gram bacteria and 12 isolates were negative Gram bacteria. Test result of LAB cell form showed 19 of the 84 isolates were in oval form (22.6%) and 65 isolates were in tube form (77.4%). Lactic Acid Bacteria was in oval or tube form positive Gram bacteria, did not form spore, able to ferment carbohydrate, negative catalase, and microaerophilic (Axel's son 2004). In the testing of morphology characteristic from 96 isolates, 84 isolates were of positive Gram bacteria. It was followed by physiology characterizing.

Physiology characteristic testing consisted of resistance to temperature and pH. It showed 78 of 84 isolates (92.8%) were resistant to high temperature and 6 isolates (7.1%) were not resistant to high temperature. Surono (2004) said that one of factors affected bacteria's growth was temperature. Elgadi et al. (2008) said that resistance of 14 isolates from fresh milk could grow at 45ºC of temperature. According to El Soda et al. (2003), termophylic *lactobacilli* and *cocci* groups could grow at 45ºC of temperature, but could not at 10ºC of temperature. Mesophylic group could not grow at 45ºC but could grow at 10ºC of temperature. Mesophilic *lactococci* could grow at 10ºC but could not grow at 45ºC of temperature. *Enterococci* group could grow at 45ºC and 10ºC of temperature.

Resistance test to pH resulted in 70 isolates (89.7%) of 78 could grow at 4.5 of pH and 8 isolates (10.3%) could not grow at that pH. According to Fowoyo & Ogunbanwo (2010), isolate type of lactic acid which

lived at 2 of pH was fewer. This type of bacteria had better ability to life in very acidic condition, and able to produce bigger organic acid number. Organic acid produced could be used to improve aroma, texture, and flavor of fermented products. In the physiology characteristic testing, 70 of 84 isolates were resistant to high temperature and low pH. It was followed by biochemistry characteristic testing.

Result of biochemistry characteristic testing showed that in testing of production of CO_2 from glucose to 70 isolates of indigenous LAB of North Sumatra river buffalo milk showed that 21 isolates (30%) could produce CO_2 (heterofermentative), whereas 49 isolates (70%) did not produce CO_2 (homofermentative). Abdulah & Oman (2010) also reported similar result that homofermentative LAB number was found more in cow milk, cheese, and fermented milk. According to Axelsson (2004), based on fermentation pattern, LAB was divided into 3 groups, namely: homofermentative obligative, heterofermentative facultative, and heterofermentative obligate LAB.

Catalase testing showed that 52 of 70 isolates (74.3%) did not produce O_2 vesicle, so that it was grouped into negative catalase bacteria, whereas 18 isolates (25.7%) produced O_2 vesicle and grouped into positive catalase bacteria. Catalase testing was aimed to determine catalase enzyme presence in the culture of bacteria starter. Catalase enzyme production could be known by dripping H_2O_2 on bacteria preparate. If there was gas vesicle, it showed that the bacteria released O_2 and grouped into positive catalase bacteria. Bacteria that did not release the O_2 vesicle showed that the bacteria had peroxidase enzyme which could prevent O_2 production and it was stated as negative catalase bacteria (Surono 2004).

Testing result of production of dextran from sucrose to 52 isolates of indigenous LAB of North Sumatra river buffalo milk showed that 4 isolates (7.7%) produced dextran positively, whereas 48 isolates (92.3%) did not produce dextran. It was concluded that the LAB isolates which did not produce dextran did not included into *Leuconostoc* group. One of characters of the *Leuconostoc* is dextran production visualized as mucoid. Dextran was defined as water soluble polygosacaride formed from α1-6 glucosides with proportion of 0-20% (Sarwat et al. 2008).

Testing of 48 isolates in producing NH_3 from arginine showed that isolates (14.6%) produced NH_3 positively, whereas 41 isolates (85.4%) did not produce NH_3. Isolates that produced NH_3 did not selected to further testing because by producing NH_3, it was feared will affect the product aroma. Tserovska et al. (2002) said that 60% of LAB from cheese and milk able to produce NH_3 from arginine.

Research result of LAB identification by biochemistry characteristic testing showed that it was obtained 40 of 70 LAB isolates which meet LAB characteristics, so that it was continued to further test. The further test was probiotic characterizing to evaluate LAB resistance to low pH. LAB 41st characteristic identified successfully was presented in Table 1.

LAB resistance testing of low pH

LAB isolates resistance to low pH was one of probiotic conditions, because stress of probiotic LAB was started at the time the LAB exposured by acidity condition of the gastric. According to Wildman & Medeiros (2000), gastric had around 2 of pH. Resistance testing of low pH was needed to determine capability of LAB culture of isolates of river buffalo milk to survive to the gastric acidity.

Figure 1, shows LAB population before and after exposured to low pH which experienced a population decline of 1.24±0.68 log cfu/ml. In other study, in LAB isolate from buffalo milk in India showed just 2 of 3 isolates tested that survived at 2 of pH for 3 hours incubation, whereas 1 isolate only survived for 1.5 hours (Shafakatullah & Chandra 2014). In the testing of LAB isolate from breast milk, all of the isolates experienced number cell decreasing with different decreasing value to each isolate with decreasing range around 0.57-7.24 log cfu/ml (Nuraida et al. 2011). Resistance testing at low pH of LAB isolate from goat milk showed that all of the tested isolates experienced population decrease less than 1 log cfu/ml (Setyawardani et al. 2014).

Ability to survive in better acidic condition for each isolate was showed by different cell number change of all tested isolates. According to Nuraida (2011), isolate ability to survive at this acidic condition was *strain dependent*. This was due to cytoplasm membrane difference of each bacteria. The difference affected characteristic and permeability of membrane.

Under acidic condition, LAB could maintain acidity of cytoplasm, so that protein and enzyme in the cell could still work optimally. LAB isolate was adaptable in low pH because it had an internal cell pH regulation. This can be achieved by existence of enzyme synthesis and producing proton (H+) from inside of the cell that was occurred through ATP hydrolysis (H+-ATPase) process. The Lactic Acid Bacteria was survive from acid damage because the existence of histidine decarboxylase and arginine of deaminase enzyme. LAB tolerance of high acidity was caused by its capability to maintain more alkaline pH of cytoplasm than extracellular pH (Pan et al. 2011).

Result showed of North Sumatra river buffalo milk that had the best resistance or experienced lowest population decrease were isolate L12, L16, L17, L19, L20, M10, P8, S3, S19, and S20. The 10 of that isolates were selected to be tested of LAB species using API test CHL 50.

Table 1. Identification of LAB isolates from North Sumatra river buffalo milk

Isolate Code	Catalase	Gram	Cell Form	pH 4,5	Temperature (45ºC)	CO_2 Production.	NH_3 Production	Dextran Production
L7	-	+	tube	+	+	+	-	-
L8	-	+	tube	+	+	-	-	-
L11	-	+	tube	+	+	+	-	-
L12	-	+	tube	+	+	-	-	-
L13	-	+	tube	+	+	-	-	-
L14	-	+	oval	+	+	+	-	-
L15	-	+	oval	+	+	-	-	-
L16	-	+	tube	+	+	+	-	-
L17	-	+	tube	+	+	+	-	-
L18	-	+	oval	+	+	+	-	-
L19	-	+	tube	+	+	+	-	-
L20	-	+	tube	+	+	-	-	-
M1	-	+	tube	+	+	-	-	-
M2	-	+	oval	+	+	+	-	-
M3	-	+	tube	+	+	-	-	-
M10	-	+	tube	+	+	-	-	-
M11	-	+	tube	+	+	-	-	-
M12	-	+	oval	+	+	+	-	-
M14	-	+	oval	+	+	-	-	-
P1	-	+	tube	+	+	+	-	-
P2	-	+	tube	+	+	-	-	-
P4	-	+	tube	+	+	-	-	-
P5	-	+	tube	+	+	+	-	-
P6	-	+	tube	+	+	-	-	-
P7	-	+	tube	+	+	+	-	-
P8	-	+	oval	+	+	-	-	-
P9	-	+	tube	+	+	+	-	-
P10	-	+	tube	+	+	+	-	-
P11	-	+	tube	+	+	-	-	-
P13	-	+	tube	+	+	-	-	-
P14	-	+	oval	+	+	+	-	-
S3	-	+	tube	+	+	-	-	-
S6	-	+	tube	+	+	+	-	-
S9	-	+	tube	+	+	-	-	-
S10	-	+	tube	+	+	-	-	-
S11	-	+	tube	+	+	+	-	-
S12	-	+	tube	+	+	+	-	-
S13	-	+	oval	+	+	-	-	-
S14	-	+	oval	+	+	+	-	-
S19	-	+	oval	+	+	-	-	-
S20	-	+	tube	+	+	-	-	-

L = Lubuk Pakam
M = Medan
P = Patumbak
S = Siborong-borong
\+ = There is a growth
\- = There is no growth

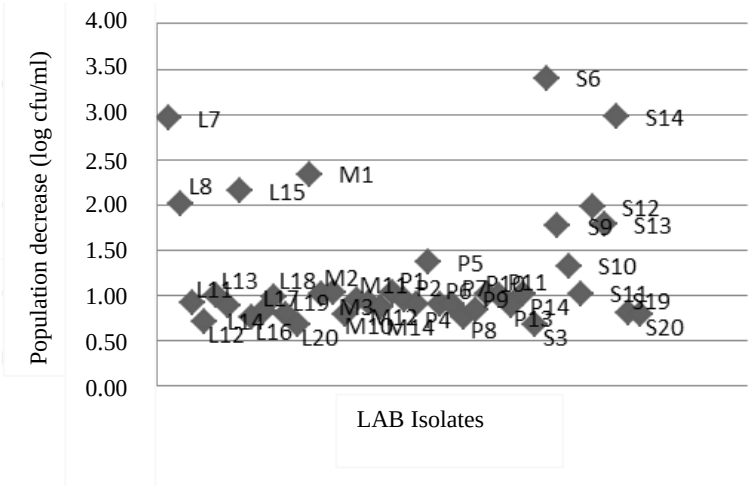

Figure 1. Population decrease of LAB after it exposured by low pH

Table 2. Identification of LAB species using API test CHL 50

Code of isolate	Name of LAB species	Percentation (%)
L12	*Lactobacillus pentosus*	94.4
L16	*Lactobacillus brevis*	96.6
L17	*Lactobacillus brevis*	96.6
L19	*Lactobacillus brevis*	99.5
L20	*Lactobacillus plantarum*	99.9
M10	*Lactobacillus plantarum*	99.9
P8	*Lactococcus lactis*	96.2
S3	*Lactobacillus plantarum*	99.9
S19	*Lactococcus lactis*	96.2
S20	*Lactobacillus plantarum*	98.3

L= Lubuk Pakam; M= Medan; P= Patumbang; S= Siborong-borong

LAB identification using API test CHL 50

Identification of LAB species using API test CHL 50 was done to the best 10 isolates based on resistance testing to low pH. It was presented completely in Table 2. Testing by API test CHL 50 resulted in 4 LAB species, namely: *Lactobacillus plantarum* (4 isolates), *Lactobacillus brevis* (3 isolates), *Lactobacillus pentosus* (1 isolate) and *Lactococus lactis* (2 isolates).

L12 isolate was identified as *Lactobacillus pentosus* with acid level of 94.4%, isolate L16, L17, and L19 were identified as *L. brevis* with acid level of 96.6%; 96.60%, and 99.50%, respectively. Isolate L20, M10, S3, and S2 were identified as *L. plantarum* with same acid level of 96.20%. Based on genotyping testing result by analysis of gene 16 sequences of S rRNA and after alignment of nucleotide sequence from selected isolate (isolate S3) on GenBank database showed that S3 isolate had similarity to the isolates of *L. plantarum* strain JCM 1149 with access code NCBI BLAST NR. 117813.1 with similarity level of 99% (unpublished data). This result showed that isolation and identification of API test CHL 50 was in line with the genotyping sequence of gene 16 S rRNA.

Testing result of species identification using API test CHL 50 of isolates from buffalo milk in India showed that there were 6 LAB species, namely: *L. bulgaricus, L. plantarum, L. lactis, L. acidophilus, L. brevis*and *L. rhamnosus* (Tambekar et al. 2009; Singh & Sharma 2009; Azis et al. 2009; Syafakatullah et al. 2014). LAB species testing of processed buffalo milk (cruds) in Indonesia showed that there were 6 LAB species, namely: *L. brevis, L. plantarum, L. casei, L. paracasei, Lactococcus lactis, Leuconostoc mesenteroides* (Surono 2003), whereas Sunaryanto & Mawoto (2012) identified successfully one of LAB species, namely: *L. plantarum*.

CONCLUSION

In this research 41 LAB isolates from North Sumatra river buffalo milk were isolated and identified. They survived successfully in low pH. Identification result of species tested by API test CHL 50 showed that from selected 10 isolates, 4 species were identified successfully, namely: *Lactobacillus plantarum, L. brevis, L. pentosus* and *Lactococus lactis.*

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Effectivity of Probiotic, Micromineral Enriched Yeast and Their Combination with *Azadirachta indica* Leaves Containing Tannin on Fermentability and Digestibility of *Pennisetum* hybrid

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ABSTRAK

Sofyan A, Sakti AA, Karimy MF, Julendra H, Istiqomah L, Herdian H, Damayanti E, Suryani AE. 2014. Efektivitas probiotik, mikromineral terkorporasi khamir yang dikombinasikan dengan daun *Azadirachta indica* mengandung tanin terhadap fermentabilitas dan kecernaan *Pennisetum* hybrid. JITV 20(2): 95-104. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1164

Eksplorasi imbuhan pakan organik untuk ternak saat ini terus dilakukan untuk menggantikan antibiotik sebagai pemacu pertumbuhan. Probiotik dari bakteri asam laktat telah digunakan secara luas untuk mendukung keseimbangan mikroba saluran pencernaan. Selain itu, mineral organik ditambahkan pada pakan untuk meningkatkan ketersediaan dalam mencegah kelainan metabolisme akibat defisiensi mineral. Penelitian ini bertujuan untuk mengevaluasi penambahan probiotik (*Pediococcus acidilactici* RS2) dan mikromineral terkorporasi khamir/*micromineral enriched yeast* (MEY) yang dikombinasikan dengan tanin dari daun mimba (*Azadirachta indica*) terhadap fermentabilitas rumput Raja (*P.* hybrid) dengan menggunakan teknik produksi gas *in vitro*. Perlakuan terdiri dari P0 (kontrol/hijauan tanpa imbuhan), P1 (P0+MEY); P2 (P0+MEY+tanin); P3 (P0+Probiotik); P4 (P0+Probiotik+MEY), dan P5 (P0+Probiotik+MEY+tanin) yang disusun dalam rancangan acak lengkap dengan 3 ulangan setiap perlakuan. Suplementasi MEY (P1), probiotik+MEY (P4) dan probiotik+MEY+tanin (P5) nyata (P<0,05) meningkatkan produksi gas hijauan tanpa berpengaruh pada asam lemak volatil (VFA), jumlah protozoa, produksi metana dan kecernaan *in vitro*. Produksi gas tertinggi tercatat pada hijauan yang diberi perlakuan P4 diikuti P5, P1, P2, P3 dan kontrol. Pengaruh perlakuan terhadap kinetika produksi gas menunjukkan perbedaan yang nyata pada inkubasi setelah 8 jam. Walaupun perlakuan hanya berpengaruh terhadap kinetika produksi gas (b, c dan total gas), berdasarkan analisis kluster-hirarki menunjukkan beberapa parameter yang terdiri dari asetat, propionat, kecernaan *in vitro*, jumlah protozoa dan produksi gas metana sangat berkorelasi dengan parameter kinetika produksi gas. Dapat disimpulkan bahwa fermentabilitas hijauan dapat ditingkatkan dengan suplementasi mineral organik maupun kombinasinya dengan probiotik maupun probiotik+tanin tanpa berpengaruh negatif terhadap kecernaan *in vitro*.

Kata Kunci: Fermentabilitas, Kecernaan *In Vitro*, Mineral Organik, Probiotik, Tanin

ABSTRACT

Sofyan A, Sakti AA, Karimy MF, Julendra H, Istiqomah L, Herdian H, Damayanti E, Suryani AE. 2015. Effectivity of probiotic, micromineral enriched yeast and their combination with *Azadirachta indica* leaves containing tannin on fermentability and digestibility of *Pennisetum* hybrid. JITV 20(2): 95-104. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1164

Organic additive for animal had been explored to replace antibiotic growth promoter. Probiotic from lactic acid bacteria was widely used to support the microbial balances in digestive tract, while organic mineral was added into diets to improve bioavailability for preventing mineral deficiency disorders. This experiment was aimed to assess probiotic (*Pediococcus acidilactici* RS2) and micromineral enriched yeast (MEY) combined with tannin from neem (*Azadirachta indica*) leaves containing tannin on king grass (*P.* hybrid) fermentability using *in vitro* gas production technique. Treatments consisted of P0 (control/forage without additive), P1 (P0+MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY), and P5 (P0+Probiotic+MEY+crude tannin). The study was arranged in a completely randomized design (CRD) with three replications in each treatment. Probiotic, MEY or tannin supplementation significantly increased (P<0.05) gas production without affecting volatile fatty acid, protozoa numbers, methane production and *in vitro* digestibility of forage. The highest cumulative gas production was found in forage treated by P4 followed by P5, P1, P5, P2, P3 and control. Kinetic of gas production was significantly affected by treatments after 8 h incubation. Although the treatments were only significantly affected gas production kinetic (*b*, *c* and total gas), the hierarchical cluster analysis indicated that some parameters consisted of acetate, propionate, *in vitro* digestibility, protozoa numbers, and methane production were closely correlated to the gas production kinetic parameters. It was concluded that either organic mineral supplementation or its combination with probiotic, and probiotic+tannin improved fermentabilty of forage without negative effect on *in vitro* digestibility.

Key Words: Fermentability, *In Vitro* Digestibility, Organic Mineral, Probiotic, Tannin

INTRODUCTION

Ruminant contribute to meat and milk supply for human consumption. Low quality feedstuffs is the biggest constrain in ruminant productivity. Supplementation of feed additive such as antibiotic growth promoters (AGP) had been reported could improve the nutrient utilization and animal performance. However, feed additive containing AGP had been banned since January 2006 in accordance with pathogenic resistant risk and antibiotic residues in animal products and by-products (EC 2012).

Feed additive based on organic material had been explored to replace antibiotic in order to support the ruminant performance. Probiotic from lactic acid bacteria are widely used to support the microbial balances in digestive tract. Probiotic is microorganism that supports animal health through maintaining and improving the intestinal microbial balance and its immune response. It is necessary to consider this probiotic, because rumen constitutes an effective animal-microbe interdependency system from that each partner derives benefit to the host animal (Arokiyaraj et al. 2014). The role interdependency system was played by complex microbial community in which the domains bacteria, archaea, and eukarya that responsible for degrading fiber particle from forage as major energy sources (Fraga et al. 2014).

Many species of lactic acid bacteria were used as probiotic cultures for ruminant such as *Lactobacillus animalis*, *L. paracasei*, *Bacillus coagulans* (Agazzi et al. 2014), *Pseudobutyrivibrio ruminis* (Fraga et al. 2014) and *Pediococcus acidilactici* (Cobos et al. 2011; Arokiyaraj et al. 2014). Moreover, Cobos et al. (2011) revealed that *P. acidilactici* isolated from rumen lamb could survive in low pH condition (4.71-4.98) without causing negative effect on dry matter digestibility. It implies that *P. acidilactici* can be used as probiotic.

On the other side, productivity of ruminant was influenced by mineral deficiencies. Although diets was formulated based on the nutrient requirement, in some cases, ruminant metabolic disorder caused by mineral deficiency. Haenlein & Anke (2011) reviewed that mineral deficiency is not only affect productivity parameters but also causes serious disease in ruminant. Organic mineral was added into diets to improve bioavailability for preventing mineral deficiency disorders. Artificial organic mineral (Selenium) involved yeast fermentation had been reported by Gresakova et al. (2013) that Se-yeast had higher absorption in digestive tract and body cell than Se-inorganic form. In addition, Rabiee et al. (2010) reviewed that complex trace mineral in organic form increased ruminant productivity and health.

The other strategy to optimize feed digestibility is through methane reduction by tannin. Neem tree (*Azadirachta indica* A. Juss) is tropical plant, its leaves contain secondary compound such as flavanoid and tannin (Pandey et al. 2014; Bhatta et al. 2015). Many researchers reported that plant secondary metabolites such as tannin could be used to modify ruminal fermentation in which improving feed utilization through reducing methane emission and therefore tannin is considered as a natural compound possessing methane mitigating effect (Bodas et al. 2012; Jayanegara et al. 2012).

Nutrition management strategy for enhancing ruminant production by supplementation of organic feed additive consisting of probiotic and organic mineral was necessary done. The availability of mineral in digestive tract affects the rumen microbe activity. This research was conducted to evaluate addition of probiotic (*Pediococcus acidilactici* RS2) and micromineral enriched yeast (MEY) combined with leaves containing tannin (*A. indica*) on king grass (*P.* hybrid) fermentability using *in vitro* gas production technique.

MATERIALS AND METHODS

Sample preparation

King grass (*Pennisetum* hybrid) was harvested on 70 days after previous cutting (harvest) and used as forage sample, then chopped and dried in oven at 60°C up to reach 12-14% of moisture content. The sample was ground and sieved into two mm particle size. Similarly, this procedure was applied in preparing leaf of Neem (*Azadirachta indica* A. Juss) which was used as tannin source.

Probiotic lactic acid bacteria (*P. acidilactici* RS2) was isolated from cattle rumen and prepared by spray-drying method. Isolate was facultative anaerobically cultivated in deMann Rogosa Sharpe (MRS) Broth media for 18 h at 37°C. The culture was centrifuged at 4500 rpm for 10 minutes, then the pellet/biomass was mixed with the sterilized skim solution (20% w/v) and gum arabic (1% w/v). The solution was homogenized using the digital homogenizer at 8000 rpm for 5 minutes. The culture solution was dried into powder form using the spray dryer (Lab Plant SD-Basic). Spray dryer operating conditions as follows: inlet air temperature was 110°C, outlet air temperature was 55-60°C, and the speed of pump was '3' speed unit. The dried culture viability was evaluated by spread plate method and adjusted to 10^9 cfu/g of bacterial cell density.

Micromineral enriched yeast (MEY) was produced by inoculating *S. cerevisiae* ATCC 9763 into media fortified by micro minerals consisted of Fe, Mn, Cu, Co, Zn, and I. Cassava (*Manihot* sp.) flour as a substrate for fermentation, and the formulation per kg substrate

consisted of $FeCl_2.4H_2O$ (0.177 g), $MnCl_2.4H_2O$ (7.129 g), $CuSO_4.5H_2O$ (9.810 g), $ZnSO_4.7H_2O$ (12.646 g), $CoCl_2.6H_2O$ (0.192 g), and KI (0.217 g). Fermentation was conducted for 7 days in facultative condition then dried in oven at 55°C (up to 24-48 h, DM 10%), followed by ground and sieved into one mm particle size.

Fermentability and *in vitro* digestibility assessment

The sample and rumen liquid were prepared prior to *in vitro* assessment. Two ruminally fistulated Ongole crossbreed cattle adapted by feeding forage (*P*. hybrid) and concentrate (80:20 in dry matter basis) were used as rumen liquor donor. Rumen fluid was taken using aspirator, and immediately transported in pre-warmed vacuum flask (39ºC water temperature) and filtered.

In vitro fermentability was evaluated using *in vitro* gas production technique according to Menke & Steingass (1988). Gas production kinetics was calculated based on the exponential equation according to Ørskov & McDonald (1979). The exponential equation is $P = a+b\ (1-e^{-ct})$ with describing *P* is total gas production, *a* is the gas production from soluble fraction, *b* is the gas production from insoluble fraction, *c* is the rate of gas production, *t* is the time of incubation and *e* is *Euler's* constant (2.7183…). The estimated value of *a*, *b* and *c* were calculated by a fitting curve method using Neway Software developed by Chen (1997). This study was conducted based on Completely Randomized Design (CRD) with six treatments and three replications. Nutrient composition from each treatment was showed in Table 1. The treatments were described as follows:

P0 = control/forage 380 mg (dry matter=92.5%)
P1 = P0+3% micromineral enriched yeast (MEY)
P2 = P0+3% MEY+2% crude tannin
P3 = P0+0.1% probiotic (10^9 cfu/g)
P4 = P0+3% MEY+0.1% probiotic
P5 = P0+3% MEY+ 2% crude tannin+0.1% probiotic

Fermentation was conducted in 100 mL syringe glass (Fortuna model, Poulten and Graft Gmbh Germany). Two syringes containing rumen-buffer without sample (blank) was used in the experiment. All of syringes consisted of samples and blank were randomly incubated for 48 hours in an incubator at 39°C.

Table 1. Nutrient composition of diets consisting of king grass supplemented by either MEY, probiotic or crude tannin

Nutrients	Treatment diets					
	P0	P1	P2	P3	P4	P5
DM	92.5	92.6	92.5	92.5	92.6	92.5
CP (%DM)	11.7	11.4	11.7	11.7	11.4	11.7
CF (%DM)	25.5	25.2	24.8	25.5	25.2	24.8
EE (%DM)	2.1	2.0	2.1	2.1	2.0	2.1
NFE (%DM)	45.0	46.0	46.1	45.0	46.0	46.1
CT (%DM)	-	-	0.28	-	-	0.28

P0 = *P*. hybrid;
P1 = P0+Micromineral Enriched Yeast/MEY;
P2 = P0+MEY+crude tannin;
P3 = P0+Probiotic;
P4 = P0+Probiotic+MEY;
P5 = P0+Probiotic+MEY+crude tannin;
DM = dry matter;
CP = crude protein;
CF = crude fiber;
EE = ether extract;
NFE= nitrogen free extract;
CT = condensed tannin
Nutrient composition was calculated by reference consisted of King grass (Rumiyati 2006); *A. indica* (Obun et al. 2013; Bhatta et al. 2015);
DM content was confirmed by re-analysing in our experiment

Cumulative gas production was recorded at 0, 1, 2, 4, 6, 8, 12, 18, 24, 36 and 48 hours. After 48 h incubation gas was released and the fluid contained in syringe was taken for analysis of protozoa population, VFA, and *in vitro* dry matter and organic matter digestibility. Dry matter (DM) and organic matter (OM) of substrate from each syringe was measured according to AOAC (2012) method. Percentage of DM or OM differences between initial and after incubation and corrected with blank were calculated as *in vitro* digestibility as followed the formula:

$$\text{IVDMD} = \frac{[DMf - (DMr - DMb)]}{DMf} \times 100\ \%$$

and

$$\text{IVOMD} = \frac{[OMf - (OMr - OMb)]}{OMf} \times 100\ \%$$

Where IVDMD: *in vitro* dry matter digestibility, IVOMD: *in vitro* organic matter digestibility, DM*f*: dry matter of feed, DM*r*: dry matter of residue, DM*b*: dry matter of blank, OM*f*: organic matter of feed, OM*r*: organic matter of residue, and OM*b*: organic matter of blank.

Analysis of volatile fatty acid, methane, and counting protozoa

Volatile fatty acid (VFA) product from fermentation was measured according to Sun et al. (2013). Sample was prepared by addition of meta-phosphoric acid and stored at -20°C before analysis. Analysis of VFA was performed by gas chromatography (Shimadzu type 8A) with *packed column* GP10% SP-1200/1% H_3PO4 on 80/100 Chromosorb WAW (Supelco, Bellefonte, PA).

Analysis of methane (CH_4) from gas production was conducted after 18 h of incubation. Ten ml of gas from each sample was taken using vacuum syringe then injected into a vacuum tube. Gas samples were analyzed using gas chromatography (Shimadzu GC-14B) completed with ProparakQ Column (50°C) with helium (He) carrier gas at 60 ml/min flow rate and a flame ionization detector (150°C) as described by Duan et al. (2013). The number of protozoa was counted using hemocytometer and stained with methyl green formalin saline/MFS. MFS solution contained 100 mL of 35% formaldehyde solution, 900 mL of distilled water, 0.6 g of methyl green and 8.0 g of NaCl as described by Sun et al. (2013).

Data analysis

Variables measured were *in vitro* digestibility (IVDMD and IVOMD), fermentability (gas production kinetics, *a*, *b* and *c*), individual volatile fatty acids (VFA), non-glucogenic ratio (NGR), protozoa numbers, methane production (CH_4) and CH4/OMD ratio. The protozoa cell number was converted to logarithmic transformation. Data were evaluated by analysis of variance (ANOVA) and the differences among mean treatments were analyzed using post hoc test of Duncan's Multiple Range Test performed by the CoSTAT statistical software (Cohort 2008).

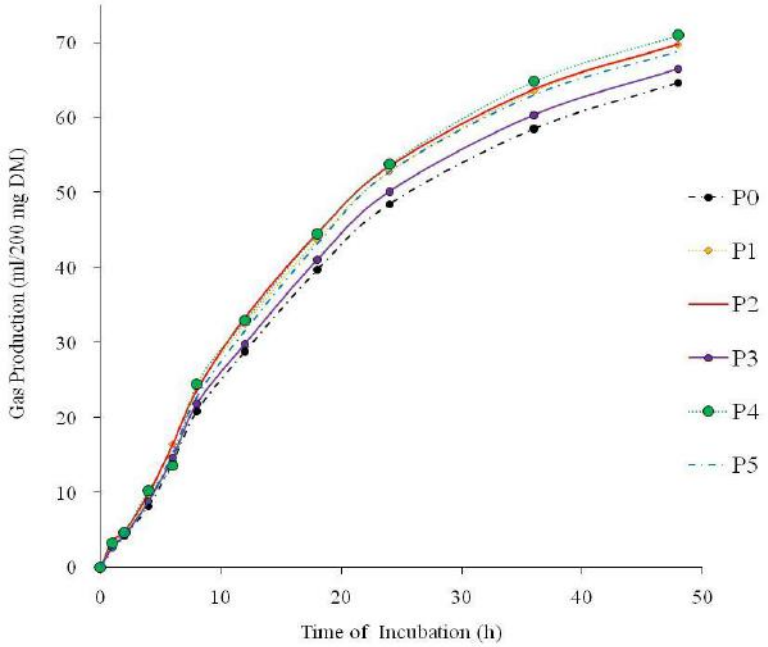
Analysis of interrelationship parameters was performed by hierarchical clustering analysis/HCA (Ametaj et al. 2010). Visualization of HCA was performed using dendogram-heatmap that constructed using `heatmap.2` function from `gplots library` in the R-statistical software (R Core Team 2013). Pre-treatment data were calculated based on the relative differential data from treatments and control using fomula x_t/x_o-1 where $x_t$and x_o denote treatment and control data respectively.

RESULTS AND DISCUSSIONS

Ruminal fermentability characteristics evaluated by *in vitro* gas production were showed on Table 2. Total gas, gas production rate (*c*) and gas production from insoluble fraction (*b*) of forage was significantly affected by treatments ($P<0.05$). The other parameters consisted of gas production from soluble fraction (*a*) or total fraction (*a*+*b*) from forage had tendency ($P\lesssim0.11$) affected by treatments. Forage was supplemented by probiotic+MEY (P4) generated the highest gas production compared to the others.

Gas production generated from the soluble fraction (*a*) resulted negative value that indicated ruminal microbes need adaptation time (*lag* phase) before degrading the insoluble particle. The previous study reported by Arhab et al. (2010) that negative value could be interpreted as *lag* time of ruminal microbes to degrade soluble fraction and then to adhere to cellulosic fraction.

Production of total volatile acid, acetate (C2), propionate (C3), butyrate (C4) and non glucogenic ratio (NGR) indicated no significant difference ($P>0.05$) among treatments (Table 2). Total VFA was varied between 43 ~ 62 mM per mL of rumen fluid. Production of VFA from forage treated by P1, P3, P5, and P4 were 43.2%, 26.2%, 19.3% and 7.7% higher than P2 and control (P0). Non glucogenic ratio seemed constant in all treatments with average value 4.42 except for P5 treatment (13% higher than control), however proportion of C2:C3:C4 was constant about 70:20:10 for all treatments. Based on the ruminal fermentation stoichiometry, individual VFA proportion consisting of C2, C3 and C4 were 60-70%, 20-30% and 10-15% respectively (Wolin et al. 1997).



P0 (*P.* hybrid); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin)

Figure 1. Cumulative gas production of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin incubated during 48 h

In the present study, *in vitro* dry matter (IVDMD), organic matter (IVOMD) digestibility and production of volatile fatty acid (VFA) were not influenced by treatments either probiotic or combination with MEY and tannin. The similar result was also reported by Sun et al. (2013), that probiotic *Bacillus subtilis* supplementation on total mix ration (TMR) did not affect the dry matter digestibility during 24 h, moreover it decreased significantly ($P<0.05$) neutral detergent fiber (NDF) digestibility. In contrast, Fraga et al. (2014) studied that addition of probiotic consisted of *Pseudobutyrivibrio ruminis* significantly increased total VFA concentration of wheat straw. It seems that probiotic supplementation affected *in vitro* digestibility might be influenced by culture adaptation associated with complexity in ruminal ecosystem.

Compared to other animal, supplementation of feed additive complex containing chelated/organic mineral, probiotic, yucca extract indicated that no significant improvement of feed digestibility in horse (Gordon et al. 2013). This varying result could be caused by many factors such as age of animal, viability of probiotic culture, and initial mineral status of the animals.

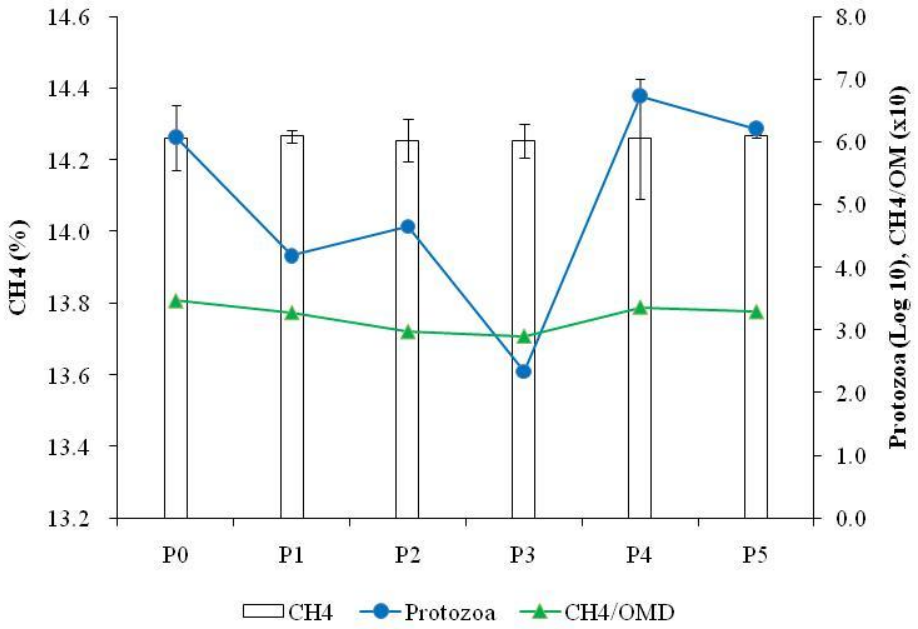
Fermentability evaluated by *in vitro* gas production was indicated by kinetic gas production parameters. Based on the kinetic curve of gas production, all treatments did not significantly influence gas production up to 8 hours of incubation. However, after 8 hours incubation, gas production from forage was significantly affected by treatment (Figure 1). The highest cumulative gas production found at forage treated by P4 followed by P5, P1, P5, P2, P3, and control. It was closely related to the kinetics parameter (*a*, *b*, and *c* values) which indicated that gas production in probiotic treatment and control was lower than others (Table 2).

Methane emission from ruminant reflected the energy lost. The amount of methane production conversely indicated the nutrient utilization. Methane (CH_4) production and methane corrected by organic matter digestibility (CH_4/OMD) during incubation showed similar result in all treatments, around 14.05±0.35% and 32.11±4.25% respectively. CH_4 and CH_4/OMD clearly tended to decrease on treatments P2 and P3.

Table 2. Gas production kinetic, volatile fatty acid production and *in vitro* digestibility of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin

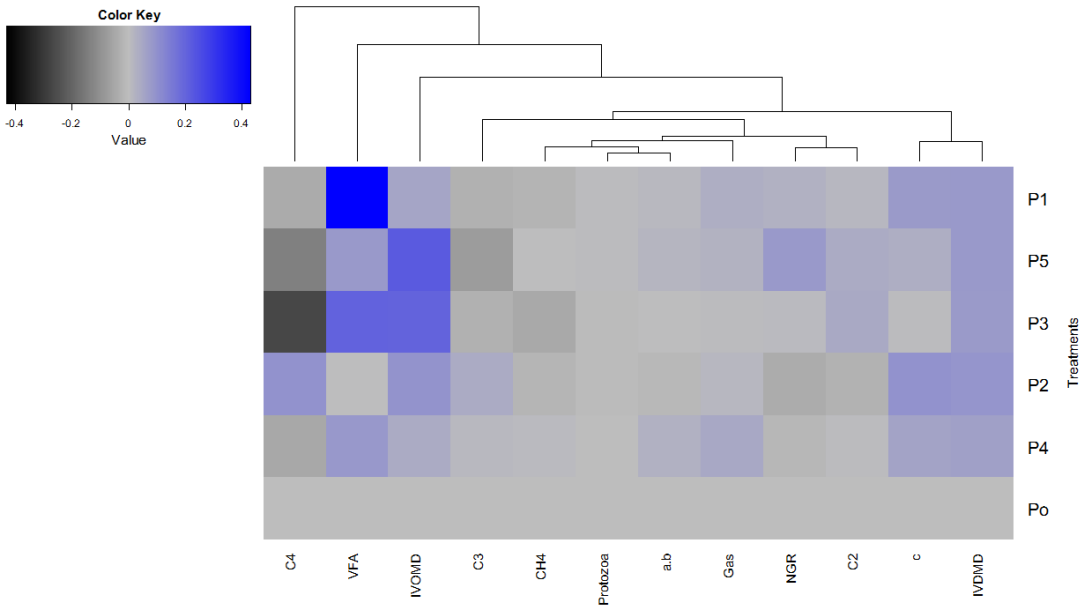
Variables	Treatments						P-value
	P0	P1	P2	P3	P4	P5	
Fermentability/Gas production kinetic parameters							
a (mL)	-0.68±0.27	-0.81±0.14	-0.65±0.29	-0.76±0.15	-0.66±0.10	-1.09±0.06	0.097
b (mL)	47.52±0.61^{ab}	48.12±1.27^{ab}	46.92±0.80^{b}	47.72±0.16^{ab}	48.87±0.48^{a}	48.86±0.67^{a}	0.046*
c (mL/h)	0.042±0.002^{b}	0.046±0.001^{a}	0.047±0.002^{a}	0.043±0.001^{b}	0.045±0.001^{ab}	0.044±0.001^{ab}	0.036*
a+*b* (mL)	46.84±0.35	47.32±1.38	46.28±1.01	46.95±0.08	48.21±0.49	47.76±0.67	0.111
Gas (48h) (mL)	40.25±0.57^{d}	41.71±0.69^{ab}	40.92±0.56^{bcd}	40.39±0.16^{cd}	42.22±0.42^{a}	41.24±0.27^{bc}	0.002*
Volatile fatty acids							
Acetate (mM)	30.55±16.18	43.82±7.36	29.66±11.64	39.28±1.37	33.43±17.34	36.80±10.78	0.684
Propionate (mM)	8.64±4.32	12.10±1.69	8.96±2.82	10.80±0.23	9.12±4.03	9.40±2.93	0.691
Butyrate (mM)	3.97±1.54	5.88±1.27	4.56±1.59	4.39±1.58	3.94±2.04	5.30±2.73	0.587
Total VFA (mM)	43.16±22.03	61.80±10.27	43.19±15.80	54.48±1.39	46.49±23.40	51.50±16.37	0.703
NGR	4.44±0.14	4.58±0.18	4.27±0.28	4.45±0.15	4.38±0.52	5.01±0.16	0.308
In vitro digestibility							
IVDMD (%)	39.40±1.68	42.64±2.82	43.10±3.27	42.58±4.84	41.95±1.02	42.70±1.31	0.638
IVOMD (%)	41.11±1.61	43.46±2.95	45.11±4.33	49.50±12.33	42.80±0.86	50.39±12.09	0.586

P0 (*P*. hybrid); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin). Gas production from soluble fraction (*a*); and insoluble fraction (*b*); rate of gas production (*c*); IVDMD: *In vitro* Dry Matter Digestibility; IVDMD: *In vitro* Organic Matter Digestibility; * Significant Difference



P0 (*P.* hybrid); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+ MEY+crude tannin)

Figure 2. Protozoal cell number, methane production, and methane per organic mater digestibility (CH_4/OMD) of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin



VFA: volatile fatty acids; C2: acetate; C3: propionate; C4: butyrate; CH4:methane; NGR: non-glucogenic ratio; IVDMD: *in vitro* dry matter digestibility; IVDMD: *in vitro* organic matter digestibility; P0 (*P. hybrid*); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin). Color key: (0) denotes no change; (+) denotes value of proportion increase; (-) denotes value of proportion decrease

Figure 3. Dendogram-heatmap visualized hierarchical-clustering analysis of altering ruminal VFA, protozoa, methane production, fermentability, and *in vitro* digestibility

Moreover, protozoa number appeared similar in all treatments was around 6.05 Log10 per mL rumen fluid (Figure 2). Supplementation of probiotic *Propionibacterium* could not alter methane production (Alazzeh et al. 2014). Indeed, Vyas et al. (2014) recommended to combine probiotic supplementation with concentrate to increase molar proportion of propionate and subsequently reduced the CH_4 emissions.

Probiotic from lactic acid bacteria could reduce methane production by the mechanism of formate utilization in rumen, in turn formate would be used by methanogenic bacteria as methane precursor (Jeyanathan et al. 2014). In this study, inability probiotic to reduce methane might be correlated with constraint adaptability of culture in ruminal ecosystem. Similarly, Alazzeh et al. (2014) revealed that probiotic have no effect on methane reduction could be associated with adaptability with other ruminal microbes and including utilization of methane precursor. Moreover, probiotic also did not influence number of protozoa. Both of probiotic and protozoa indicated no antagonistic relationship as similar previously reported by Qadis et al. (2014).

Supplementation of tannin from *A. indica* leaves did not affect the methane production and protozoa number. It might be caused by low dosage of tannin (0.28%) used in the present study. Currently, Bhatta et al. (2015) reported that addition of 2.5% *A. indica* leaves (contained 13.8% of condensed tannin) did not reduced methane production and protozoa number, however the methane production was inhibited when the addition was increase to 25%.

Interrelationship parameters evaluated by hierarchical cluster analysis (HCA) indicated that proportion change of IVOMD, VFA and C4 were higher independently changed by treatment than other parameters (cluster I). Other parameters were categorized into cluster II (C3, CH4, protozoa, *a*+*b*, gas), cluster III (IVDMD and *c*), cluster IV (C2 and NGR). Cumulative gas production and IVDMD had higher similarities related to the change of treatment (Figure 3.). The tree clusters and their shorter *Euclidean* distance indicated higher similarities. Similarity between two metabolites or parameter was represented by branch height (Ametaj et al. 2010).

Organic matter digestibility of forage treated by P5 (probiotic+MEY+tannin) and P3 (probiotic), then total VFA from P1 reflected the higher increased differences than others while forage treated P4 (MEY+tannin) was not affect VFA and gas production. This result revealed that forage treated by probiotic either combining with organic mineral or tannin affected ruminal fermentation. Improving ruminal fermentation by feed additive containing lactic acid bacteria was previously reported Hillal et al. (2011). Otherwise, presence of tannin in P2 (MEY+tannin) resulted in VFA and fermentability lower than P1 might attribute to characteristic of condensed tannin in *A. indica* could inhibit ruminal microbes. Previously, Seresinhe et al. (2012) reported supplementation of condensed tannin could reduce forage fermentability by indicating gas production.

Methane production was closely related to the protozoa number. In the rumen, methane was generated by *Archaea bacteria* that consumed hydrogen. Archea activity was closely symbiosis with protozoa (Bhatta et al. 2015) and endosymbiotic between protozoa and *Archaea* responsible to methane formation (Belanche et al. 2015). Change of NGR was closely related to C2, C3 and C4 in which C2 was dominantly affected due to 60% of VFA. Moreover, gas production and degradation rate of particle affected the IVDMD. Sandoval-Castro et al. (2002) studied the relationship between digestibility and production gas parameter. Significant correlation between gas production and digestibility were influenced by many factors consisted of nutrient composition in which associated microbial ability to adhere and degrade the fraction of feed materials.

Overall, *in vitro* digestibility indicated nutrient utilization in the rumen which was attributed by gas production kinetic parameters and others parameters consisting of VFA, protozoa and methane production. In this study, presence of probiotic complemented by mineral organic or crude tannin had a positive effect on ruminal fermentation of forage.

CONCLUSION

Supplementation of feed additive contained micromineral enriched yeast (MEY) in combination with probiotic *P. acidilactici* RS2 or probiotic+*A. indica* leaves improved fermentability of king grass without affected volatile fatty acid, protozoa numbers, methane production, and *in vitro* digestibility.

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Productivity of *Calliandra calothyrsus*, *Indigofera zollingeriana* and *Gliricidia sepium* on Acid Soil in the Greenhouse

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ABSTRAK

Herdiawan I, Sutedi E. 2015. Analisis produktivitas tanaman *Caliandra calothyrsus*, *Indigofera zollingeriana* dan *Gliricidia sepium* pada lahan kering masam di rumah kaca. JITV 20(2): 105-114. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1165

Tanah masam umumnya kurang baik bagi budidaya tanaman, tidak terkecuali untuk leguminosa pohon karena mengandung Al^{3+} dan Mn^{2+}. Kedua mineral tersebut mungkin bersifat toksik bagi pertumbuhan dan produksi tanaman *Caliandra calothyrsus*, *Indigofera zollingeriana*, dan *Gliricidia sepium* yang umumnya digunakan sebagai hijauan pakan. Penelitian ini bertujuan untuk membandingkan daya toleransi dan produktivitas ketiga jenis tanaman pada tanah masam. Tiga jenis tanaman ditanam di rumah kaca dengan media tanam tanah Ultisol dengan pH 4,57 yang diambil dari perkebunan kelapa sawit Sei-Putih, Medan. Percobaan menggunakan 3 jenis tanaman sebagai perlakuan dan diulang sebanyak 12 kali dengan menggunakan Rancangan Acak Lengkap (RAL). Data dianalisis dengan sidik ragam (ANOVA) menggunakan metode SPSS, program exel, dilanjutkan dengan uji LSD apabila terdapat data yang berbeda nyata. Peubah yang diamati adalah morfologi tanaman, konsentrasi Al^{3+} pada jaringan tanaman, tinggi tanaman, diameter batang, jumlah percabangan pada batang, panjang akar, produksi tanaman, kandungan nutrisi, energi, dan kecernaan *(in-vitro)*. Konsentrasi Al^{3+} pada daun, batang dan akar nyata paling tinggi ditemukan pada *G. sepium*, sedangkan konsentrasi terendah pada *I. zollingeriana. G. sepium* tumbuh lebih kerdil, diameter batang tidak berbeda dengan *C. calothyrsus*, tetapi keduanya lebih rendah dari *I. zollingeriana*. Jumlah percabangan pada *I. zollingeriana*, nyata lebih tinggi dibandingkan dengan *G. sepium*. Panjang akar *C. calothyrsus* tidak berbeda nyata dengan *I. zollingeriana*, sedangkan akar *G. sepium* lebih pendek. Nodulasi akar hanya terbentuk pada *I. zollingeriana.* Produksi biomasa dan kandungan protein tertinggi, serta nilai kecernaan terbaik dicapai pada *I. zollingeriana.* Berdasarkan data kandungan Al^{3+} pada jaringan daun, batang dan akar *I. zollingeriana* nyata lebih toleran terhadap tanah masam *(Ultisol).* Daya toleransi tanaman berpengaruh terhadap pertumbuhan, produksi biomasa, kandungan nutrisi, dan nilai kecernaan yang lebih baik.

Kata Kunci: Avian Influenza, *Cleavage Site*, Hemaglutinin, Patogenitas, Filogenetik

ABSTRACT

Herdiawan I, Sutedi E. 2015. Productivity *of Calliandra calothyrsus, Indigofera zollingeriana* and *Gliricidia sepium* on acid soil in the greenhouse. JITV 20(2): 105-114. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1165

Acid soil which contains Al^{3+} and Mn^{2} is generally unfavorable for crop including the tree legumes. The minerals are toxic to the plants resulted minimalization of growth and crop production. *Caliandra calothyrsus*, *Indigofera zollingeriana*, and *Gliricidia sepium* were tree legumes those are generally used for forage. The aim of this study was to compare their tolerancy to Al^{3+} and growth production on acid soil. The plants were grown in ultisol soil with 4.57 of pH collected from Palm Oil plantation, Sei-Putih, Medan. The experiment was carried out using completely randomized design (CRD) with kind of plants as the treatment and 12 times replication. The data were analyzed by ANOVA using the SPSS and excel program, followed by LSD test when the data was significantly difference. Variables measured were plant morphology, concentration of Al^{3+} in the plant tissues, plant height, stem diameter, number of stem branches, root length, plant production, nutrient content, energy and in vitro digestibility. The highest Al^{3+} contents in leaves, stem and root were significantly observed in those *G. sepium*, while the lowest contents was observed from those of I. zollingeriana. G. sepium was the most dwarf plant and its stem diameter was comparable with the one of *C. calothyrsus*, but was lower than that of *I. zollingeriana*. The highest number of branches was significantly observed in *I. zollingeriana*, while the lowest one was at *G. sepium*. The root length of *C. calothyrsus* was comparable with that of *I. zollingeriana*, while *G. sepium* root was the shorthest one. Root nodulation was only formed at *I. zollingeriana*. The highest biomass production was observed at *I. zollingeriana* which also had highest protein content and the best digestibility. Data from Al^{3+} concentration in tissues of leaves, stems and roots showed that *I. zollingeriana* was the most tolerant plant to acid soils. This tolerancy also affected higher plant growth, biomass production, nutrient concentration, and digestibility.

Key Words: Avian Influenza, Cleavage Site, Hemagglutinin, Pathogenicity, Phylogenetic

INTRODUCTION

Large acid dry soil potential in Indonesia is a chance to produce various crop commodities (food crops, estates, or livestock's feed crops). Several soils that generally had acid pH in the dry area were *Entisols*, *Inceptisols*, *Ultisols*, *Oxisols*, and *Spodosols*, especially for area, which has wet climate with high rainfall. The largest ordos were Ultisol and Inceptisols, with its dominan spreading was in the Sumatera, Kalimantan, and Papua (Mulyani et al. 2004). Utisol was one of soil types which widely spreaded reaching 45794000 ha or about 25% of total of Indonesian land, which was widely used as estate area, among other oil palm, rubber, and industry plantation (Subagyo et al. 2004).

Concentration of high alumunium in the form of Al^{3+} was one of limiting factors of crops cultivation on the acid (pH ≤5.5) land which inhibited crop's growth and production (Gergichevich et al. 2010). In line with it, Sopandie (2006) said that reactive alumunium (Al^{3+}) was released from soil in the form of $AI(OH)^{2+}$ and $AI(OH)^{3+}$ which often become toxic to all agricultural crops, because of the AI ion inhibited root growth quickly in concentration of micromolar. Kinraide & Hagerman (2010) also said that alumunum was very strong toxic to the crop and would inhibit their growth, decrease biomass production and overall crop yield. Ryan & Delhaize (2010) said that Al^{3+} toxicity in acid land (pH ≤5.5) was main factor of stress to the crop, especially to the root tissue of crop that directly contacted with the environtment (Rizonsphere). According to Rengel & Zhang (2003), decreasing of root growth was one of early and very clear simpthom of AI toxication in micromolar (µM) concentration limit which boosted the decreasing of water and nutrient absorption capacity. The alumunium able to inhibit essential nutrient absorption to the crops such as Ca, Mg, Mn, Fe, Mo, and P (Poschenrieder et al. 2008).

Mora et al. (2006) said that AI toxicity changed physiology and biochemistry process of the crop, and its consequence affected its productivity. In despite of Al inhibited process of metabolism and crops growth, but until a certain threshold, tolerant crops (Utama et al. 2005) could tolerate AI effect. According to Wang et al. (2006), several crops were tolerant to aluminum stress because they eliminated AI, so that was not toxic and affected growth and productivity of the crops. Based on Polania et al. (2010), in the genotype of the tolerant crops showed better rooting performance and expected would produce higher biomass. In the context of the sustainable forage on the acid dry land, it needed acid-tolerant foragrs. Several forages included in *Fabaceae* family had good enough tolerance to the dry acid land (Tjelele 2006). *C. calothyrsus*, *I. zollingeriana*, and *G. sepium* were tree leguminous which could be used as forage in the acid soil of estate area, so that needed to be observed extent to which its tolerance and productivity.

MATERIALS AND METHODS

This research was carried out at greenhouse of Agrostology, Ciawi Indonesian Research Institute for Animal Production (AIAT) using 3 tree leguminous (*C. calothyrsus*, *G. sepium*, dan *I. zollingeriana*). Growing media used in this research was Ultiosl acid soil from oil palm plantation, Medan with chemical composition of the soil was presented in the Table 1.

Each of the three crops was planted in plastic pot (40 and 50 cm of diameter, which its base was coated by plastic with 40 cm of diameter to hold water spilled when watering. Planting process was started by seeding of the three crops on the seeding tray for 4 weeks old. After 4 weeks, the seeds were moved into small polybags until 8 weeks old and further, those seedling were moved into plastic pots which were fulfilled by 40 kg of planting media. Watering was done once of 2 days. Volume of watering was adapted with determination result of field capacity (FC). Morphology of crops and root were observed visually at the end of this study. Dry weight production of the crops was done for 44 weeks in every 90 days of harvest day using digital scale. Crops growth was measured in every 2 weeks using meter with 1 cm of scale and digital Vernier calipers. Al^{3+} concentration and nutrient composition was determined from proximate analysis in the nutrition laboratory of IRIAP.

The experiment was carried out using completely randomized design (CRD) with kind of plants as the treatment and 12 times replication. The data were analyzed by ANOVA using the SPSS and excel program, followed by LSD test when the data was significantly difference. Variables measured were plant morphology, concentration of Al^{3+} in the plant tissues, plant height (height, stem diameter, number of branches, root length), plant production (dry weight of leaves, branches and stems, biomass, and ratio of stem/leave) nutrient content (crude protein, crude fiber, fat, dust, Ca, and P), energy and in vitro digestibility of dry and organic materials.

Table 1. Analysis result of soil from oil palm plantation, Sei Putih, Medan

Composition	Soil Samples			
	A	B	C	Average
pH	4.40	4.80	4.50	4.57
Organic material (%)				
Carbon (C)	1.64	1.27	1.56	1.49
Nitrogen (N)	0.16	0.12	0.15	0.14
C/N ratio	10.02	10.06	10.04	10.04
Anion exchange rate				
Ca	7.56	6.99	7.73	7.43
Mg	1.44	2.41	1.32	1.72
K	0.57	0.48	0.43	0.49
Na	0.49	0.51	0.49	0.50
Al^{3+}	1.36	1.15	1.27	1.26

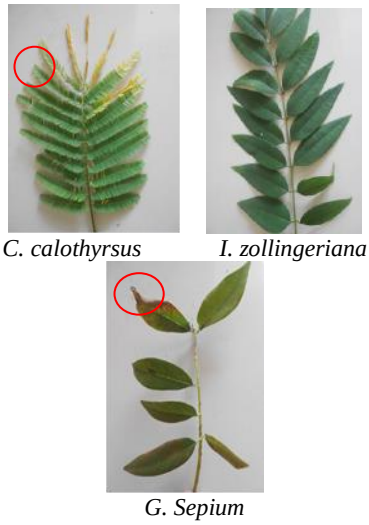
*Analyzed at Soil Laboratory, Indonesian Center for Agricultural Technology Assessment and Development (ICATAD) in 2013

RESULT AND DISCUSSION

Morphology of the crops in the acid soil

Based on observation result, morphologically, *I. zollingeriana* was better than *C. callothyrsus* and *G. sepium*, such as shave and color of leaves, stems, and the number of brunches. Stem of the *C. callothyrsus* seemed thicker than *I. zollingeriana* and *G. sepium*, likewise the leaves color of the I. *zollingeriana* seemed greener than *C. callothyrsus* and *G. sepium* which were yellowish and dry out at the tip of the leaves. *G. sepium* was stunted growth with thin stem, and in almost the leaves had tallow spot, whereas *C. callothyrsus* tree grown spindly and almost all leaves had yellow spot (Figure 1.).

Suntoro et al. (2014) said that the condition of the soil pH is low (acidic), the solubility of some minerals not available to needed for the chlorofil formation.



C. calothyrsus *I. zollingeriana*

G. Sepium

○ = yellow spot

Figure 1. Morfology of leaf in the acid soil

Consequently decreased leaf chlorophyll, leaf color yellow spots which in turn is inhibited the process of photosynthesis rate. Thus the amount of photosynthate produced is very low, this determines the lower plant growth. This showed that both of the *I. zollingeriana* and *C. callothyrsus* were poisoned by micro content. Sumarno (2005) said that clear symptoms of crops that sensitive to the acid soil were very stunted growth, brownish yellow leaves, very limited root growth, minimal flower-shaped, and minimal seed number, very low productivity or failed to produce seeds (Figure 2).

According to Wang et al. (2006) poisoned plant by AI would has nutrient deficiency, such as P, Ca, Mg, Mn, and Fe, so that morphologically was more stunted and its productivity was low. Schaberg et al. (2006) found the same thing in the sugar maple plant that showed high AI content affected low Ca and restricted plant growth. Sumarno (2005) said that the growth of soybean plant on acid soil was suffer due to abiotic and biotic stresses, such as (a) vegetative growth was hampered because of macro and micro deficiency; (b) AI or Mn poisoning; (c) nodule formation was inhibited; (d) the plant was easier to get drought stress; and (e) root growth was inhibited. Furthermore, it was said that very clear symptoms were very stunted growth, brownish yellow leaves, very limited root growth, minimal flower-shaped, and minimal seed number, very low productivity or failed to produce seeds. Although Al concentrations in the nutrient solutions are within the micromolar range (25-1,600 µM), they are sufficient to induce morphological and physiological damage in some crops, and even more significant changes in seedlings (Rengel 1996). Al-toxicity is an important stress factor for plants, limiting plant growth, development and the subsequent performance of commercial crops (Poschenrieder et al. 2008); Rout et al. 2001).

C. calothyrsus

I. zollingeria na

G. sepium

Figure 2. Morphology of the crops in the acid soil

C.calothyrsus

I. zollingeriana

G. sepium

○ = Nodules

Figure 3. Root morphology on the acid soil

Root morphology on the acid soil

Morphology chance of *C. callotyrsus* root was not clearly seen, the roots grown lengthwise, feathers grown normally, but nodule was not found in the main root or the branches. Root morphology of *I. zollingeriana* showed normal growth, the most root hair in every main root or branches and nodule was formed. Root morphology chance was occurred in the *G. sepium*, that was abnormally growth, shorter with slightly root feather and only grown at the root tip (Figure 3).

No formation of nodule on the root of *C. calothyrsus* and *G. sepium* was one indicator of the AL^{3+} poisoning consisted of root cells damage, so that root did not grow well aside from poisoning the environment (rhizosfer) that affected root microbe (rhizobium) growth.

As noted by Taiz & Zeiger (2006) that growth of crops rooting was highly depended on growth environment of the crops and its growth was controlled by crop's activity. Factors affected the soil environment among other factor of physic, biology, and chemistry of soil. The first symptom came up from AI poisoning was short rooting system as a result of cell extension inhibition (Chairani et al. 2007). So that according to Wang et al. (2006) who said that the first response of crop to Al^{3+} poisoning was root tissue damage, so that contributed to nutrient absorption decrease. Besides, AL^{3+} also gave bad effect to structure and function of leaves as photosynthesis machine and showed leaf necrosis, so that assimilation process not running optimally (Zhang et al. 2007).

The highly growth and extension of root under acid soil stress showed higher tolerance than its adaptation to acid soil (nutrient deficiency) with high aluminum content (Polania et al. 2010). Based on Atman (2006), general characteristics of acid soil were pH value of the soil less that 4; low nutrient content of soil organic matter (SOM); low of P availability and Cation Exchange Capacity (CEC) of soil; high content of Mn^{2+} and reactive aluminum (Al^{3+}) that may poison the root and inhibit nodule forming of the legumes. Sudaryono (2009) said that former coal mine land showed pH around 4.4-5.3 was indicated as acid soil, whereas 4.2-4.3 of pH was indicated as very acid soil. The decrease in root growth is one of the initial and most evident symptoms of Altoxicity at micromolar (µM) concentrations in plants (Rengel & Zhang 2003).

AL^{3+} concentration of crops tissue in the acid soil

Average Al^{3+} concentration in tissue of leaves, stems, and roots of *I. zollingeriana* was significantly lowest ($P<0.05$) than *C. callothyrsus* and *G. sepium* (Table 2). The highest Al^{3+} concentration was in the part of root tissue. This was because of the root was a part of crop tissue which directly contacted with rizosphere (acid soil), so that Al^{3+} concentration was accumulated more in the part of that tissue, whereas it was relatively low in the tissue.

Table 2. Al^{3+} concentration of tissue of the three legumes

Legume	Al^{3+} concentration (mg/kg)		
	Leaf	Stem	Root
C. callothyrsus	0.21[b]	26.71[b]	83.65[b]
I. zollingeriana	0.13[c]	14.33[c]	47.77[c]
G. sepium	0.35[a]	52.18[a]	135.51[a]

Not equal letter in the same column shows a significantly difference (P<0.05)

Poisoning symptom was seen from Al^{3+} accumulation in the *G. sepium* tissue, or this crop was not tolerance and disable to eliminate the Al^{3+} accumulation. *I. zollingeriana* and *C. callothyrsus* was able to eliminate the Al^{3+} accumulation on all tissue, so both of the crops still show good morphology character. Delhaize & Ryan (1995) said that crop which tolerance to the Al stress, was a crop which able to accumulate Al fewer, so that Al toxicity was relatively low.

In the soil containing of high aluminum saturation such as several areas in Indonesia, *G. sepium* grew poorly and had low survival. However, Nusantara (2009) said that *Gliricidia* crop was suitable for acid and marginal soils. According to Zang et al. (2007), aluminum in low concentration in soil was very helpful to the growth and would be toxic to the crop only when the concentration exceeds a certain threshold. Furthermore, he said that the highest threshold of the Al concentration was 800 mg/kg in the soil caused decreasing of chlorophyll content of leaves, so that assimilation process was disturbed caused crop productivity decrease. Soil used for this study was 1.26 mol or 34000 mg/kg (Table 1). Ying et al. (2006) reported that low aluminum concentration did not affect or increased the crops growth. On the contrary, Liu et al. (2006), in his study showed that surface area and dry weight of leaves of 2 soybean cultivars increased on the Al concentration treatment as much as 200 mg/kg. Furthermore, on the aluminum concentration of 200-400 mg/kg, the crops started showing assimilation rate decreasing caused by leaf stomata closing. Chen et al. (2006), states that with increasing content of Al^{3+} on the roots and leaves cause the concentration of Mg in the two organs decreases, consequently photosiyntetic active radiation (PAR) was declined.

According to Soemarno (2005), Al concentration in soil solution was very high when soil pH was low. pH value increased on waterlogged soil and Al concentration on soil solution decreased under critical level of Al poisoning. Al stress treatment at Al saturation index of 25% and 50% decreased dry weight of root of 5 soybean genotypes and increased dry weight of Wilis root. The size of the dry weight decreasing of root depended on type of genotype (Hanum et al. 2007).

Crops growth in the acid soil

Result of analysis of variance showed that *C. callothyrsus* was significantly (P<0.05) highest tree (122.47 cm) than *I. zollingeriana* (96.34 cm) and *G. sepium* (62.83 cm) in 44 weeks old (Table 3). Stem diameter of *I. zollingeriana* was significantly (P<0.05) higher by 10.21 mm compared to *C. callothyrsus* and *G. sepium* by 8.99 and 7.54 mm respectively, whereas stem diameter of *C. callothyrsus* and *G. sepium* was not significantly different. Average number of branches of *I. zollingeriana* was significantly (P<0.05) the most by 35.92 branches compared to the other crops, and the lowest was in *G. sepium* by 7.65 branches.

C. callothyrsus root was significantly (P<0.05) longer by 70.36 cm compared to *G. sepium* root by 27.19 cm, but it was not significantly different compared to the *I. zollingeriana*. According to Sumarno (2005), very clear symptoms from the crops which sensitive to acid soil were very stunted growth, tawny leaves, limited rooting growth, flower and seed number forming was minimal, very low productivity or even failed to produce seed. Silveira (2013) said that negative effect of soil acidity to forage growth generally not caused by single factor, but by several factors, which affected normally crops growth. The main factor commonly affected crops growth in the acid soil consisting of toxicity of Hydrogen ion (H^+), aluminum, mangan and essential nutrient deficiency such as phosphor, magnesium, and micronutrient.

Table 3. Growth of the three legumes in the acid soil in 44 weeks old

Legume	*Growth Parameter*			
	High (cm)	Stem diameter (mm)	Average number of branches	Root length (cm)
C. callothyrsus	122.47[a]	8.99[b]	15.88[b]	70.36[a]
I. zollingeriana	96.34[b]	10.21[a]	35.92[a]	69.54[a]
G. sepium	62.83[c]	7.54[b]	7.65[c]	27.19[b]

Not equal letter in the same column shows a significantly difference (P<0.05)

Aluminum was one of soil elements which able to cause poisoning to surrounding plants environment and inhibited the crops growth (Timotiwu 2010). In line with that, Hadiatmi (2002) said that clear symptoms in the shorgum were stunted growth, dwarf, thicker leaves and were dark green with outskirts purplish leaves or dried. Growth of crops rooting very depended on environment and controlled by activity of the crops. Daniel (2011) said that characteristics of aluminum toxicity symptom included of root defects such as thickened, twisted, short root tip and lateral roots, brown root, and did not have a good branching in rooting system.

According to Rout et al. (2001), Al caused disruption of cell fission on root cap and lateral root, cell rigidity through formation of pectin crosslink on the cell wall, and reduced DNA replication through increasing of double chain rigidity. Haling et al. (2011) said that growth and development of big and long crop root under acid land stress showed that capability of tolerance and adaptation to the acidity and saturation of high Al. The first and most recognized effect of Al-toxicity in plants is the inhibition of the division and elongation of meristematic cells and thereby the reduction in root growth (Panda et al. 2003). In line with that, Yoichiro & Midori (2011) said that length root was tolerance indicator of the crops to stress level of aluminum poisoning. Tolerant crops to aluminum would grow well, whereas root of sensitive crops would grow shorter and thick.

Crops production in the acid soil

Dry weight production of *I. zollingeriana* leaves was significantly (P<0.05) higher by 19.23 g/crop compared to *C. callothyrsus* and *G. sepium* by 15.30 and 9.37 g/crop, respectively (Table 4). Dry weight production of *C. callothyrsus* branch/stem was significantly (P<0.05) higher by 13.39 g/crop than G. sepium by 10.20 g/crop, but dry weight production of branch/stem of *I. zollingeriana* and *C. callothyrsus* was not different.

Dry weight production of *I. zollingeriana* biomass was significantly (P<0.05) higher by 32.06 g/crop compared to *C. callothyrsus* and *G. sepium* by 28.70 and 19.58 g/crop, respectively. Leaves/stems ratio of *I. zollingeriana* was significantly (P<0.05) higher by 3.44 compared to *C. callothyrsus* and *G. sepium* by 1.59 and 1.23, respectively. Generally, dry weight production of *I. zollingeriana* was highest than *C. callothyrsus* and *G. sepium*. Chen (2006) and Dewi et al. (2010) said that Al toxicity was the main factor which inhibited crop's productivity in various acid soil throughout the tropics and subtropics. According to Chen et al. (2005b), aluminum stress caused closure of stomata which was responsible to decreasing of CO_2 intake, so that the assimilation rate decrease. It affected decreasing of crop production drastically.

Ma et al. (2002) said that high Al concentration could disturb soybean growth and damage the rooting, so that absorption of nutrient and water was not optimal and caused low productivity of the crop. Based on Hilman et al. (2004), in the acid land, phosphate (P) availability became the major obstacle to increase. Type of the soils was toxic to crops and needed treatments. At pH ≤5.5, Al-toxicity is the main stress factor for plants which limits crop production (Ryan & Delhaize 2010) legume production. Haling et al. (2011), good crop performance under stress of acid soil and drought was caused by capability to tolerate the stress which was implemented in biomass production of canopy and root which was connected with acquisitions level of nutrient and water. Chen et al. (2005a) said that Al decreased CO_2 intake useful in the assimilation process of tangerines (*Citrus rehhni*), which affected to enzyme activity involved in Calvin cycle. The disruption of the assimilation cycle due to the Al induction caused decreasing of nutrition supply to the crop and decreased the production and quality of crop, especially to the sensitive crop. According to Lynch (2013), tolerant crops showed better rooting performance and it was expected would produce higher biomass. Al-toxicity results in alterations of the physiological and biochemical processes of plants and consequently their productivity (Mora et al. 2006).

Nutrition content and digestibility value of the legumes in the acid soil

Crude protein content (Table 5) of *I. zollingeriana* was significantly (P<0.05) highest by 21.80% compared to *C. callothyrsus* and *G. sepium* by 16.80 and 16.64 respectively.

Table 4. Average production per harvest of the three legumes in the acid soil

Legume	Dry weight production (g/crop)			Ratio
	Leaves	Stems	Biomass	Leaves/stems
C. callothyrsus	15.30[b]	13.39[a]	28.70[b]	1.59[b]
I. zollingeriana	19.23[a]	12.83[a]	32.06[a]	3.44[a]
G. sepium	9.37[c]	10.20[b]	19.58[c]	1.23[c]

Not equal letter in the same column shows a significantly difference (P<0.05)

Table 5. Nutrient content and digestibility value of in vitro of the three legumes

Legume	Nutrient content						Gross Energy (Kcal/kg)	Digestibility value (%)	
	CP (%)	CF (%)	CFat (%)	Ash (%)	Ca (%)	P (%)		DMD	OMD
C. callothyrsus	16.80^{b}	30.98^{a}	4.06^{a}	4.18^{b}	0.42^{b}	0.14^{b}	4.472^{a}	59.89^{c}	54.54^{b}
I. zollingeriana	21.80^{a}	23.14^{b}	3.59^{b}	6.62^{a}	1.17^{a}	0.35^{a}	4.184^{b}	73.75^{b}	76.22^{a}
G. sepium	16.64^{b}	23.08^{b}	4.38^{a}	6.08^{a}	0.75^{b}	0.14^{b}	4.162^{b}	78.02^{a}	76.88^{a}

Not equal letter in the same column shows a significantly difference (P<0.05)
CP = Crude Protein
CF = Crude Fiber
CFat = Crude Fat
DMD = Dray Matter Digestibility
OMD = Organic Matter Digestibility

In line with Yayneshet et al. (2009) who said that crude protein content of the forage on the semi-acid land in Ethiopia was decrease drastically caused by stress of drought and soil acidity. Binding of Al^{3+} to cell membrane phospholipids and transport proteins, reduces the net negative membrane surface charge, permitting the movement of anions and restricting that of cations (Huang et al.1992). The highest fiber content was reached by *C. callothyrsus* by 30.98% followed by *I. zollingeriana* and *G. sepium* by 23.14 and 23.08% respectively.

Higher content of structural component (NDF, ADF, and ADL) was found during dry season, especially in the acid soil possibility was caused by lignification height and stadium of crop maturity (Hussain & Durrani 2009).

Ash content of *I. zollingeriana* was significantly (P<0.05) different with *C. callothyrsus* but significantly not different with the *G. sepium*. Similarly, Ca and P content of *I. zollingeriana* was significantly (P<0.05) different with *C. callothyrsus* and *G. sepium*, but Ca and P content of *C. callothyrsus* was significantly not different with *G. sepium*. As said by Zhao et al. (2009) that ash level referred to mineral content closely related to soil condition, soil type, fertilizing and irrigation. Furthermore, Silveira (2013) said that negative effect of soil acidity to forage growth, generally not caused by single factor, but by several factors affected normally crop growth. The main factor generally affected crop growth in the acid soil included hydrogen ion (H^{+}) toxicity, aluminum, mangan, and deficiency of phosphor, magnesium, and micronutrient. Al content could inhibit absorption of essential nutrient, such as Ca, Mg, Mn, Fe, Mo, and P (Poschenrieder et al. 2008). According to Silveira et al. (2011) optimum absorption of the most soil nutrient was occurred when the soil pH was close to neutral. Availability of several macronutrients (N, P, K, S, Ca, and Mg) decreased as an effect of soil acidity increase, so that lime application in the acid soil tended to increase nutrient availability. Al3+ is known to affect cell membrane structure and permeability by blocking the Ca^{2+} channels (Plieth 2005).

Yamamoto et al. (1992) said that inhibition of root growth and development due to Al^{3+} poisoning, in the long term could cause decreasing of capability to absorb the nutrient, suffering from nutrient (P, Ca, Mg, or Fe) deficiency, so that caused bad effect to the growth and development of the canopy. According to White & Broadley (2003), Ca played important role as nutrient in the crops. As a divalent cation, Ca played role as structural wall and cell membrane participated in root and stem growth. Ca deficiency because of Al^{3+} content would affect crop production. Rout et al. (2001) mentioned that Al-induced effects in leaves resemble P deficiencies.

Gross energy value of *C. callothyrsus* was significantly (P<0.05) higher by 4472 kcal/kg than *I. zollingeriana* and *G. sepium* by 4184 and 4162 kcal/kg respectively. According to Dewhurst et al. (2009), gross energy increase of the forage was always in line with dry matter increase, especially to organic matter. Varela de Arruda & Fernandes (2014) said that there was a significant interaction between digestibility of dry material (DM) and gross energy (GE) of the forage which was affected metabolism energy value. Furthermore, it was said by Khachatur (2006) that total content of dry matter of grass that experienced abiotic stress decreased in line with the stress level, as well as its gross energy content.

Digestibility of *G. sepium in vitro* was significantly (P<0.05) highest by 78.02% compared to *I. zollingeriana* and *G. sepium* by 73.75 and 59.89% respectively. Furthermore, digestibility of *in vitro* organic matter of *G. sepium* was significantly not different with *I. zollingeriana* (76.88 vs 76.22), but it was significantly (P<0.05) higher than *C. callothyrsus* (54.54%). Digestibility value of *in vitro* dry matter was the number of dry matter, which could be digested and not excreted in the form of fesses, and it was assumed as absorbed part by the animal (Chuzaemi & Bruchem 1990). According to González & Hanselka (2002),

digestibility value of organic matter of the forage, from wet season to dry season experienced significant decreasing in line with concentration increase of several fiber-forming components. Based on Nisa et al (2004), digestibility value of grass and legume, generally experienced a decreasing by age increase of the plant and soil water content due to concentration increase of crude fiber in the crop tissue, lignification increase, and leaves/stems ratio decrease. Mora et al. (2006) reported that high concentration of Al^{3+} correlated with poor quality of pasture and the higher risk was body weight gain decrease of the animals.

Based on analysis test of nutrient content, all of the legumes planted on the acid soil experienced decreasing from normal condition. Average content of Crude protein of *C. calothyrsus* by 20.0, 23.1, and 25.7% respectively (Tangendjaja et al. 1991; Tangendjaja et al. 1992; Herdiawan et al. (2014). The smallest crude protein decrease was showed by *I. zollingeriana* or become more resistant to acid soil. This may be caused by low cation exchange capacity, so that nutrient absorption experienced small obstacles. Other possibility was a root tissue structural damage caused by Al^{3+} poisoning, so that root absorption effectivity to water and nutrient in the soil was decrease (Khan et al. 2008). Optimum absorption of partly nutrients was occurred when soil pH was close to neutral. Availability of several macronutrients (N, P, K, S, Ca, Mg) decreased as an effect of increasing of soil acidity, so that lime application in the soil acid tented to increase nutrient availability to corn crop (Baligar et al. 1997). It has been reported that Al inhibits the absorption of nutrients, especially Ca, Mg, Fe and Mo and less available P (Poschenrieder et al. 2008).

CONCLUSION

Al^{3+} concentration of *I. zollingeriana* was lower than *C. calothyrsus* or that crop was tolerant to acid soil. Conversely, *G. sepium* was not tolerant causing low growth and productivity. AL^{3+} effect was also seen on root morphology, where nodule forming was only occurred on *I. zollingeriana*. *C. callothyrsus* root was longer with more root hairs resembling *I. zollingeriana*, whereas *G. sepium* root was shorter and the root hair was fewer. *C. callothyrsus* was more tolerant to Al^{3+} than *G. sepium*. Crop height measurement showed that *C. calothyrsus* was highest, but the stem diameter and the number of the highest branches was found on *I. zollingeriana*. The highest biomass was found on *I. zollingeriana*, whereas the fewer biomasses were found on *G. sepium*. Data analysis of nutrient value also showed that *I. zollingeriana* was tolerant to the acid soil and could be developed in that environment.

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Molecular Analysis of Hemagglutinin Gene of Avian Influenza Viruses Isolated in 2012-2013

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ABSTRAK

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Virus Avian Influenza (AI) masih menjadi penyebab utama kasus kematian unggas di Indonesia dan di seluruh dunia. Penelitian ini bertujuan untuk menentukan patogenitas serta melakukan analisis filogenetik dan jarak kekerabatan gen hemaglutinin virus-virus AI yang diisolasi di Indonesia pada tahun 2012–2013 di Jawa Barat, Jawa Tengah, dan Medan. Sampel diperoleh dari peternakan ayam yang mengalami wabah AI dan diinokulasi serta dipropagasi dalam Telur Ayam Berembrio (TAB) Specific Pathogen Free (SPF). Cairan alantois yang dipanen 5 hari setelah inokulasi diuji terhadap aktivitas hemaglutinasi. Cairan alantois dengan hemaglutinasi positif diuji lebih lanjut untuk menentukan subtipe hemaglutinin dan neuraminidase dengan real-time reverse transcription polymerase chain reaction (RRT-PCR) dan dilakukan persiapan untuk sekuensing menggunakan reverse transcription polymerase chain reaction (RT-PCR). Sekuen gen hemaglutinin dianalisis terhadap susunan asam amino di daerah cleavage site dan jarak genetik serta hubungan kekerabatan antar virus. Hasil penelitian mengindikasikan bahwa semua isolat virus tergolong ke dalam HPAI dengan pola asam amino daerah cleavage site QRESRRKKR dan QRERRRKR. Enam isolat termasuk subtipe H5N1 sedangkan 3 isolat lainnya merupakan H5Nx. Semua isolat memiliki hubungan genetik yang dekat dengan jarak genetik kurang dari 0.3 antara virus yang satu dengan yang lainnya dan juga terhadap beberapa isolat virus AI yang menyebabkan wabah-wabah sebelumnya di Indonesia.

Kata Kunci: Avian Influenza, *Cleavage Site*, Hemaglutinin, Patogenitas, Filogenetik

ABSTRACT

Kurniasih SW, Soejoedono RD, Mayasari NLPI. 2015. Molecular analysis of hemaglutinin gene of Avian Influenza viruses isolated in 2012-2013. JITV 20(2): 115-125. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1166

Avian Influenza virus (AIV) still plays as a major cause of the death in poultry in Indonesia and around the world. The aim of this research was to determine the pathogenicity and to analyze the phylogenetic and genetic distances of hemagglutinin gene of isolated AI viruses in Indonesia in 2012-2013 particularly from West Java, Central Java, and North Sumatra. Samples were obtained from poultry farms that suffered from AI outbreaks, were inoculated and propagated in ten days old specific pathogen free (SPF) embryonated chicken eggs. Harvested allantoic fluids at 5 days after inoculation were tested for hemagglutination activity. Positive allantoic fluids were further tested to determine the hemagglutinin and neuraminidase subtype using real-time reverse transcription polymerase chain reaction (RRT-PCR) and to be prepared for sequencing using reverse transcription polymerase chain reaction (RT-PCR). The sequence of hemagglutinin genes were analyzed for the amino acid pattern of the cleavage site region and the genetic distances and relationships of those viruses. The result indicated that all of the isolates are classified as HPAI with the pattern of cleavage site regions are QRESRRKKR and QRERRRKR. Six isolates are classified as H5N1 and 3 isolates are H5Nx. All of the isolates have close genetic relationship with the genetic distances less than 0.3 between one to another and also with several AI viruses that caused previous outbreaks in Indonesia.

Key Words: Avian Influenza, Cleavage Site, Hemagglutinin, Pathogenicity, Phylogenetic

INTRODUCTION

Avian Influenza (AI) disease is potentially very harmful in poultry industry in Indonesia. In spite of the fact that farms had been vaccinated to prevent the outbreak, AI was sporadically still occurred in several areas in Indonesia, event in the poultry that had carried out routine vaccination. Not only in chicken, AI was also reported in ducks and the other waterfowls (Andesfha et al. 2013; OIE 2014).

Indonesian AI viruses are classified in cluster 2.1. Generally, there are 2 clusters of AI virus in the world, that are cluster 1 and cluster 2. Differences in cluster or subcluster may cause in differences of the antigenic structure between one and another virus, therefore vaccine which was used to prevent AI outbreak is

different in one area to another. Phylogenetic analysis is very important to determine the spreads of the virus and genetic distances between AI viruses, so that the prevention and vaccination strategy could be established to prevent the upcoming AI outbreaks (Nidom et al. 2012; Wibawa et al. 2012).

The AI virus belongs to the Orthomyxoviridae family. This virus has 8 gene segments encoding 10 viral proteins. Two types of protein which have important role in pathogenicity of AI virus are hemagglutinin (HA) and neuraminidase (OIE 2014). The HA protein functions for the attachment of the virus to the host cell and to allow fusion between virus membrane and the endosomal membrane of the host cell, whereas neuraminidase has a role in releasing virion progeny into the host cell (Susanti 2008). Hemagglutinin initiates virus infection by binding the receptor of the host cell (Dharmayanti et al. 2012). The hemagglutinin gene contains cleavage site region and other components including antigenic site, receptor binding residue, receptor binding pocket, and glycolisylation site (Susanti 2008).

Cleavage site region is amino acid sequence in hemagglutinin gene where proteases of host cell cleaves the HA0 precursor into the HA1 and HA2 subunits followed by fusion between virus envelope with endosomal membrane of host cell (Perdue 2008). The proteolytic activation of HA molecule is very important in infectivity and virulence of AI virus. The specificity of HA molecule could be the determinant factor to differentiate the pathogenicity of AI virus (Dharmayanti et al. 2012). The differences in HA molecules can be based on amino acid sequence in cleavage site region. Avirulent or low pathogenic AI virus generally has single basic amino acid or arginine (R), whereas virulent or highly pathogenic strains have polybasic amino acid or multiple arginine and lysine (K) (Susanti 2008; Hewajuli & Dharmayanti 2012). This study was aimed to determine the pathogenicity of AI virus isolated in 2012-2013 in several areas in Indonesia and to analyze the phylogenetic and genetic distances between those viruses nor with the isolate of Indonesian AI virus from previous outbreaks.

MATERIALS AND METHODS

Virus isolation

Virus isolates in this study were taken from organs of AI infected chickens (Table 1). Virus isolation was conducted based on the method of Swayne et al. (1998). Twenty grams sample were mashed and added by 80 ml PBS containing Penicillin (10000 IU/ml), Streptomycin (2000 µg/ml), Kanamycin Sulfate (650 µg/ml), and Amphotericin B (20 µg/ml). The suspension was centrifuged for 10 minutes in 5000 rpm. Supernatant obtained was used to inoculate 10 days old specific pathogen free embryonated chicken eggs with 0.2 ml inoculum per egg. The inoculated eggs were incubated in 38-39^{o}C temperature with 60-65% relatives humidity. The incubated eggs were candled to determine the mortality of embryos and dead embryos were stored in 4^{o}C temperature overninght followed by allantoic fluid harvesting. The allantoic fluids were collected into sterile tube and tested for hemagglutination activity, and then stored in -80^{o}C temperature.

Hemagglutination (HA) test

Hemagglutination test was carried out by rapid hemagglutination and microtitration hemagglutination based on Office International des Epizootis (OIE) (2014).

Virus identification by Real-time Reverse Transcription Polymerase Chain Reaction (RRT-PCR)

RNAs of the virus were extracted using QIAamp Viral RNA Mini Kit (Qiagen 2010). Identification of H5 subtype was carried out by RRT-PCR using forward primer 53 5′-ACATGCCCAAGACATACTGGAA-3′, reverse primer H5r-182 5′-GAATTCGTCACACATTGGGTTTC-3′, and probe H5 Probe-79 FAM-CACACAACGGGAAGCTCT-GCGATCT-TAMRA (Chen et al. 2007). PCR composition contained 12.5 µl 2× Quantifast Probe RT-PCR Master Mix, 1 µl primer H5f 0.8 µM, 1 µl primer H5r 0.8 µM, 1 µl probe 0.2 µM, 0.25 µl Quantifast RT Mix, 2 µl template RNA 100 ng, and 7.25 µl Rnase free-water with the total volume 25 µl. PCR process was carried out in the Qiagen Rotor-Gene Q 2plex HRM System with temperature for reverse transcription reaction was 50^{o}C for 10 minutes, initiation/activation of 95^{o}C for 5 minutes, denaturation of 95^{o}C for 15 second, and combination of annealing-extension was 52^{o}C for 60 second with 40 times of the PCR cycle. Identification of N1 subtype was carried out by the same method using forward primer N1F2 5′-GTTTGAGTCTGTTGCTTGGTC-3′, reverse primer N1R1 5′-TGATAGTGTCTGTTATTATGCC-3′, and N1-probe FAM-TTGTATTTCAATACAGCGAC-TAMRA (Payungpon et al. 2006) with annealing/extension temperature was 50^{o}C for 60 second.

Table 1. List of virus isolates 2012-2013

Isolate	Poultry	Origin	Year
A/Ck/Parung Panjang/Prl/2012	Native chicken	Tangerang	2012
A/Ck/Cigudeg/Prl/2013	Native chicken	Bogor	2013
A/Ck/Lyr.Gunungsindur/Prl/2013	Layer	Bogor	2013
A/Ck/Gunungsindur/Prl/2013	Broiler	Bogor	2013
A/Ck/Cianjur/Prl/2013	Broiler	Cianjur	2013
A/Ck/Legok/Prl/2013	Broiler	Tangerang	2013
A/Ck/Medan/Prl/2013	Broiler	Medan	2013
A/Dk/Pakijangan/Prl/2013	Duck	Brebes	2013
A/Dk/Brebes/Prl/2013	Duck	Brebes	2013

Reverse Transcription Polymerase Chain Reaction (PCR) for sequencing

Reverse Transcription-PCR reaction which was used in the sequencing process contained of 10 µl 5× Qiagen OneStep RT-PCR Buffer, 2 µl dNTP Mix 10 µM, 1.5 µl primers 0.6 µM, 2 µl Qiagen OneStep RT-PCR Enzyme Mix, 10 µl Q solution, 2 µl RNA 1-2 µg, and 21 µl RNase-free water with total volume was 50 µl. RT-PCR program consisted of reverse transcription 50^{o}C for 30 minutes, pre-denaturation 95^{o}C for 15 minutes, 40 cycles consisted of denaturation 94^{o}C for 30 second, annealing 53^{o}C for 60 second, and extension 72^{o}C for 60 second, with the final extension 72^{o}C for 10 minutes.

PCR reactions was carried out with 3 different pairs of primer including forward primer HA01 5′-TGGAGAAAATAGTGCTTCTTCTTGC-3′ and reverse primer HA645 5′-GGAAATATAGGTGGTTGGGTTTTG-3′ (Susanti 2008), forward primer HA548F 5′-CCAACCAGAGAAGGATCTTTTGG-3′ and reverse HA1215R 5′-ACTAGGCCTCAAACTGAGTGTTC-3′ (Susanti 2008), primer H5-1 5′-GCCATTCCACAACATACACCC-3′ and H5-3 5′-CTCCCCTGCTCATTGCTA-3′ (WHO 2005), following with the electrophoresis of the PCR products.

Sequencing

Purification of PCR products and sequencing process were done by PT Genetika Science Jakarta and 1st Base Malaysia. The DNA sequences were used for phylogenetic analysis and to determine the pathogenicity of the virus.

Phylogenetic analysis

Phylogenetic and genetic distance analysis among isolates were carried out by multiple alignment ClustalW in BioEdit (Alzohairy 2011). The construction of phylogenetic tree was done by MEGA 5.05 version (Tamura et al. 2011).

RESULT AND DISCUSSION

Death of embryos after AI virus inoculation

Isolate 1, 2, 4, 6, 7, and 8 caused embryo's death in 24-48 hours after the inoculation, whereas isolate 3, 5, and 9 caused embryo's dead after 48 hours (Table 2 and Table 3). The death of embryos was correlated with the virulence and pathogenicity of the virus. Swayne et al. (1998) clarified that pathogenic AI virus can cause embryo's death in 24-48 hours after 0.2 ml inoculation of the virus into allantoic cavity. The death of embryos occurred in 24-48 hours after inoculation in this study indicated that isolate 1, 2, 4, 6, 7, and 8 were pathogenic AI virus. Isolate 3, 5, and 9 caused embryo death after 48 hours. Embryo's death is related to the capability of hemagglutinin gene to be cleaved by the host cell protease. AI virus with pathogenic cleavage site but killed embryos in more than 36 hours was assumed as AI virus which had lost its pathogenicity to its native host. Parallel with the result of Kencana et al. (2014) the death of embryos as the result of non-pathogenic AI virus infection occurred in 3rd day post inoculation. This phenomenon was suspected to be occurred in isolate 3, 5, and 9. The capability of HA protein to be cleaved by host cell protease determines the spreads of

Table 2. Observation of the embryo post AI virus inoculation

<24 hours	24-48 hours	48-72 hours	72-96 hours	>96 hours
live	3 death	-	-	-
live	3 death	-	-	-
live	3 live	2 death, 1 live	1 death	-
live	3 death	-	-	-
live	3 live	2 death, 1 live	1 death	-
live	3 death	-	-	-
live	3 death	-	-	-
live	3 death	-	-	-
live	3 live	3 death	-	-
live	3 live	3 live	3 live	3 live

Table 3. The death of embryo post AI virus inoculation

Isolate	Death of embryo
A/Ck/Parung Panjang/Prl/2012	<48 hours
A/Ck/Cigudeg/Prl/2013	<48 hours
A/Ck/Gunungsindur/Prl/2013	<48 hours
A/Ck/Legok/Prl/2013	<48 hours
A/Ck/Medan/Prl/2013	<48 hours
A/Dk/Pakijangan/Prl/2013	<48 hours
A/Ck/Lyr.Gunungsindur/Prl/2013	>48 hours
A/Ck/Cianjur/Prl/2013	>48 hours
A/Dk/Brebes/Prl/2013	>48 hours

AI virus in chorioallantoic membrane of the embryo. AI virus with HA protein that capable to be cleaved by proteases has the capacity to penetrate the 3 germinal layers of the membranes and enter to the blood vessels (Lebas et al. 2013) and the visceral organs (Krauss et al. 2012). Hemagglutinin from pathogenic AI virus can facilitate the virus spreading from the allantoic space to the deeper mesenchymal vascularization layer of the chorioallantoic membrane, causes the extensive virus infection, contrary with the hemagglutinin from non-pathogenic AI virus that only causes infection in allantoic membrane and inside the allantoic cavity (Feldmann et al. 2000).

Correlation of the virus amount with hemagglutination activity and virulence of the virus

Hemagglutination test is used to determine the quantity of the virus which agglutinates the red blood cell (RBC) and it is a method which is used in the screening of the existence of a causative agent of hemagglutination such as influenza A virus in the isolates harvested from allantoic fluids. The progeny of AI virus which is released from the infected cells can be determined by hemagglutination test (Killian 2014). Agglutination of RBC by AI virus is mediated by reaction between receptor binding site of hemagglutinin molecule with sialic acid receptor of the host cell. Hemagglutinin is a part of virus which will attach on the chicken's RBC receptor causing the agglutination. The attachment would form a protoplasm bridges which eventually form a mass that precipitates in the bottom of the microplate These activities will be the basis in the hemagglutination test to determine the existence of the virus that agglutinates RBC in the allantoic fluids (Natih et al. 2010). HA titre is correlated with the amount of virus in the allantoic fluids after virus inoculation. HA titre shows negative result if the quantity of the virus is less than 10^6 embryo infectious dose (EID_{50})/ml (Kencana et al. 2014). This test is quantitatively, the value of 1 HAU is equal with 10^7 particles of the virus (Killian 2014). Positive reaction of hemagglutination test is occurred if the HA titre is valued ≥2 HAU (Koratkar et al. 2014). HA titre of the isolates in this study (Table 4) were 18-1382 HAU with the highest level was isolate 8 (1382 HAU) and the lowest one was isolate 3 (18 HAU). According to Wanasawaeng et al. (2008), chicken's embryo which was inoculated with virulent AI virus, commonly died within 32 hours with infectivity titre around 7.3-9.0 log2 HA titre or 128-512 HAU. Lang et al. (2011) mentioned that the highest HA titre existed in the allantoic fluids of the embryo harvested at the 1st and 2nd day of the AI virus isolation indicating the increasing of the newly forming infective virion. It could be assumed that isolate 1, 2, 4, 6, 7, and 8 in this study were virulent AI virus with the HA titre ranged 343-1382 HAU.

Table 4. Hemagglutination (HA) titre

Isolate	HA Titre (HAU)
A/Ck/Parung Panjang/Prl/2012	512
A/Ck/Cigudeg/Prl/2013	589
A/Ck/Gunungsindur/Prl/2013	343
A/Ck/Legok/Prl/2013	1024
A/Ck/Medan/Prl/2013	1382
A/Dk/Pakijangan/Prl/2013	512
A/Ck/Lyr.Gunungsindur/Prl/2013	18
A/Ck/Cianjur/Prl/2013	42
A/Dk/Brebes/Prl/2013	74

Moresco (2010) declared that low pathogenic AI virus showed the HA titre less than the amount that could be detected in the allantoic fluids from virus isolation, whereas Koratkar et al. (2014) said that positive reaction of the hemagglutination test occurred if HA titre was ≥2 HAU. Isolate 3, 5, and 9 had positive HA titre or valued as ≥2 HAU, but the HA titre of the isolates were low (around 18-74 HAU). It could be suspected that isolate 3, 5, and 9 were AI viruses which may have experienced a mutation or reasortion that caused the unability to replicate in the host cell to reach the appropriate hemagglutination titre or its hemagglutination activities were lower.

Subtype of avian influenza viruses isolated in 2012-2013

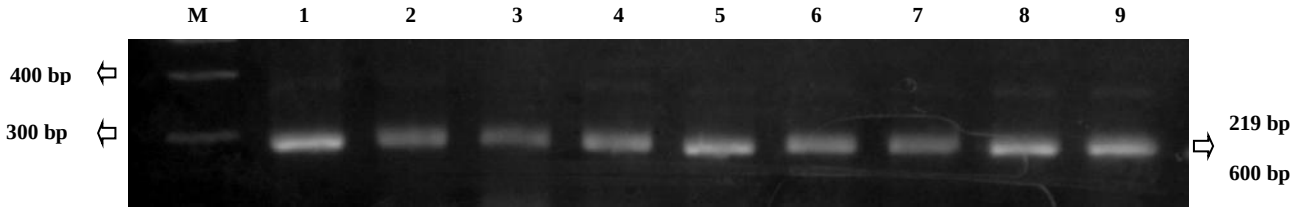
Real Time RT-PCR results threshold cycle (Ct value) which is the amount of PCR cycle at the time when fluorescence increases and can be detected significantly at the early stage of the positive samples. Threshold cycle represents the change of the cycle number at the time when the positive amplification reaction could be measured (Payungporn et al. 2006). The RRT-PCR results positive samples if the Ct value <33, whereas it is negative if Ct value >35 (SADC 2010). In this study, 9 isolates were positive H5. Six from the 9 isolates were positive N1, therefore the six isolates (isolate 1, 2, 4, 6, 7, and 8) could be classified into H5N1 subtype, whereas the other 3 isolates (isolate 3, 5, and 9) were H5 subtype aside of N1 (H5Nx subtype). The results of the RRT-PCR test were presented in the Table 5.

The results of RRT-PCR in this study showed 10^6-10^8 copies of RNA of the H5 positive isolates and 10^5-10^8 copies of RNA of the N1 positive isolates. RRT-PCR product was detected using specific sequence probe to amplify only the specific target. Specific HA probe could be used for the quantification of the virus with different subtypes in one virus mixture (OIE 2014; Spackman 2014). The amount of RNA copy that could not be read at the N1 RRT-PCR test from isolate 3, 5, and 9 indicated that there were no amplification occured, and the isolates were considered to be negative N1. However, Ct value and the amount of RNA copy did not show correlation with the result of virus isolation and hemagglutination test. The H5Nx isolates apparently had Ct value and RNA copy comparable with isolates from H5N1 subtype. This was possible because RRT-PCR test has the high sensitivity and specificity that can detect and amplify the very small quantity of RNA. The difference in AI virus detection between RRT-PCCR and virus isolation is caused by the difference of the ability of both test to detect the parts of the virus. Virus isolation only detects live virus and cannot detect virus that had been inactivated or had experienced other treatments, whereas the RRT-PCR can detect live or inactivated virus (Spackman 2014)

Table 5. Threshold cycle (Ct value) and number of RNA copy of H5 and N1 in RRT-PCR

Isolate	Ct		RNA copy /µl	
	H5	N1	H5	N1
A/Ck/P.Panjang/Prl/2012	20.02	13.90	1381543	283112136
A/Ck/Cigudeg/Prl/2013	17.22	15.16	63815531	125437247
A/Ck/Gunungsindur/Prl/2013	16.54	23.75	92686366	481532
A/Ck/Legok/Prl/2013	15.94	16.12	128404046	67493217
A/Ck/Medan/Prl/2013	16.18	15.14	112597534	126602979
A/Dk/Pakijangan/Prl/2013	17.06	17.81	6975631	22448560
A/Ck/Lyr.Gnsindur/Prl/2013	16.89	tba	76147115	tba
A/Ck/Cianjur/Prl/2013	17.85	tba	45025043	tba
A/Dk/Brebes/Prl/2013	20.54	tba	10331331	tba

[a]Result could not be read by the thermalcycler



M. Marker 1kb; 1. A/Ck/Parung Panjang/Prl/2012; 2. A/Ck/Cigudeg/Prl/2013; 3. A/Ck/Lyr.Gunungsindur/Prl/2013; 4. A/Ck/Gunungsindur/Prl/2013; 5. A/Ck/Cianjur/Prl/2013; 6. A/Ck/Legok/Prl/2013; 7. A/Ck/Medan/Prl/2013; 8. A/Dk/Pakijangan/Prl/2013; 9. A/Dk/Brebes/Prl/2013

Figure 1. PCR product of H5-1 H5-3 primer amplification

In the other side, in connection with the mortality of the embryo and HA titre, the results of the RRT-PCR test had a correlation with virus isolation and hemagglutination test. RRT-PCR test, technically accurately measured RNA quantity correlated with HA content (Spackman & Suarez 2005). Isolate 3, 5, and 9 showed embryo death after 48 hours and had lower HA titre (18, 42, and 74 HAU respectively) were H5Nx subtype, therefore could be assumed that only isolates classified in H5N1 subtype that could kill embryo in 24-48 hours and had higher HA titre (343-1382 HAU) compared to H5Nx subtype.

Amino acid sequences of the Cleavage site of hemagglutinin gene

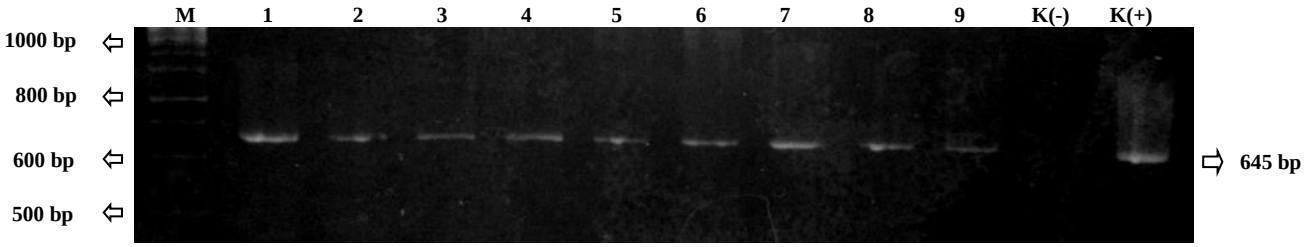
The electrophoresis of the PCR product is presented in Figure 1. Sequencing result of the PCR products with primer that specificaly amplified cleavage site region of hemagglutinin gene showed sequence of repeated arginine (R) and lysine (K) amino acid (Table 6). AI virus with polybasic amino acid sequence of arginin or lysine are classified as pathogenic (Gohrbandt et al. 2011; Li et al. 2011). Virulence characteristic of AI that distinguish HPAI from LPAI is the ability of the virus to be cleaved by proteases which could be found in almost all type of host cells. Hemagglutinin gene produced as single polypeptide is cleaved into HA1 and HA2 subunit before the virus become infectious. This cleavage process is important for the fusion domain to be active during virus replication process and facilitate AI virus infection to the host cell. Generally, cleavage process is conducted by trypsin or trypsin-like proteases which cleaved HA protein due to the recognition of single arginine. Monobasic (single arginine) amino acid sequence could be cleaved by tryptase produced by ephitelial cell of gastrointestinal and respiratory tracts, therefore the AI virus infection is restricted in gastrointestinal and respiratory organs. However, if polybasic amino acid or repeated arginine or lysine are existed, the cleavage site becomes accessible to furin or other ubiquitous proteases, such as proprotein convertase 6 (PC6) in the Golgy that is found in most

Table 6. Amino acid sequence of hemagglutinin gene on cleavage site

Isolate	Amino acid pattern
A/Ck/Parung Panjang/Prl/2012	QRESRRKKRG
A/Ck/Cigudeg/Prl/2013	QRESRRKKRG
A/Ck/Gunungsindur/Prl/2013	QRESRRKKRG
A/Ck/Legok/Prl/2013	QRESRRKKRG
A/Ck/Medan/Prl/2013	QRESRRKKRG
A/Dk/Pakijangan/Prl/2013	QRESRRKKRG
A/Ck/Lyr.Gunungsindur/Prl/2013	QRERRRKRG
A/Ck/Cianjur/Prl/2013	QRERRRKRG
A/Dk/Brebes/Prl/2013	QRERRRKRG

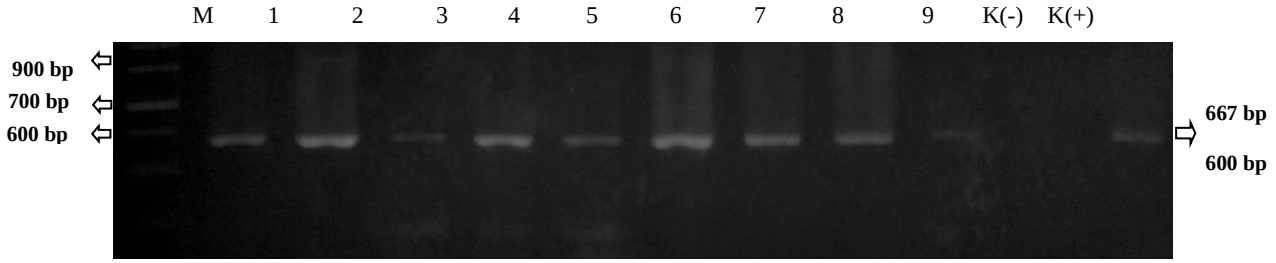
cells and so the infection could be occurred in the various tissues and infect systemically. Therefore, amino acid sequence in the cleavage site determines the pathogenicity of AI virus, as AI virus could be classified into highly pathogenic (HPAI) or low pathogenic (LPAI) (Bogs et al. 2010; Gohrbandt et al. 2011). The cleavage site region of hemagglutinin gene plays very important role in producing infectious viral progeny during the AI virus infection (Leijon et al. 2011). Polybasic amino acid or arginine and lysine in the hemagglutinin of H5N1 AI have a role in systemic infection, so that AI virus could be isolated from blood, brain, nerve, cerebrospinal fluid, cornea, heart, lungs, kidney, pancreas, intestine, caecum, and feces (Yamamato et al. 2010; Kim et al. 2015).

The research of Wibawa et al. (2011) in ducks in Central Java in 2007-2008 showed amino acid patterns of cleavage site were QRERRRKKR, QRESRRKRR, QRESRRRKR, QKESRRKKR, and QRESRRKKR. Wibawa et al. (2012) also isolated AI virus that caused outbreak in duck in several areas in Central Java, Yogyakarta, and East Java in September-November 2012 and showed amino acid patterns of the cleavage site was QRERRRKR. Research of Andhesfa et al. (2013) of birds in Central Java, Yogyakarta, and East



M. Marker 1kb; 1. A/Ck/Parung Panjang/Prl/2012; 2.A/Ck/Cigudeg/Prl/2013; 3. A/Ck/Lyr.Gunungsindur/Prl/2013; 4. A/Ck/Gunungsindur/Prl/2013; 5. A/Ck/Cianjur/Prl/2013; 6. A/Ck/Legok/Prl/2013; 7. A/Ck/Medan/Prl/2013; 8. A/Dk/Pakijangan/Prl/2013; 9. A/Dk/Brebes/Prl/2013; K(-). Negative control, K(+). Positive control

Figure 2. PCR product of HA01-HA645 primer amplification



M. Marker 1kb, 1. A/Ck/Parung Panjang/Prl/2012; 2.A/Ck/Cigudeg/Prl/2013; 3. A/Ck/Lyr.Gunungsindur/Prl/2013; 4. A/Ck/Gunungsindur/Prl/2013; 5. A/Ck/Cianjur/Prl/2013; 6. A/Ck/Legok/Prl/2013; 7. A/Ck/Medan/Prl/2013; 8. A/Dk/Pakijangan/Prl/2013; 9. A/Dk/Brebes/Prl/2013; K(-). Negative control, K(+). Positive control

Figure 3. PCR product of H548F-H1215R primer amplification

Java in 2011-2012 also explained that AI virus which successfully isolated had amino acid pattern of the cleavage site of QRESRRKKR and QRERRRKR and caused AI outbreaks in those areas.

Sequencing analysis result of the hemagglutinin cleavage site of the AI viruses in this study showed that 6 isolates (H5N1) had amino acid pattern of QRESRRKKR and 3 isolates (H5Nx) had QRERRRKR. QRESRRKKR pattern by previous scientist was reported to be found in waterfowls, whereas in this study the pattern was found in AI isolate which cause outbreaks in broiler chicken (A/Ck/Gunungsindur/Prl/2013, A/Ck/Legok/Prl/2013, A/Ck/Medan/Prl/2013), native chicken (/Ck/Parung Panjang/Prl/2012, A/Ck/Cigudeg/Prl/2013), and duck (A/Dk/Pakijangan/Prl/2013). It was assumed that AI virus that caused the outbreaks were came from ducks or other waterfowls wandering around the farm or kept in backyard which clinically was healthy and became as a source of transmission of AI virus. WHO (2011) and Coker et al. (2014) explained that wild or domestic waterfowls has important role in AI virus transmission to the other domestic birds. Wild waterfowls is a natural host of the type A influenza virus and plays important role in the ecology and virus propagation. Commonly from the natural host, the type A influenza virus could be transmitted to the other birds (Pillai et al. 2010). AI virus is replicated in epithelial cells of the intestinal track of the wild waterfowls. Virus excretion through feces could become the source of transmission of AI virus to other domestic birds. Duck's living place which is close to the water environment makes it possible to be a media of virus transmission through the water (Hewajuli & Dharmayanti 2012). When AI virus has been transmitted by duck to the other birds, there would be a high morbidity, clinical symptoms, and mortality rate (Henning et al. 2010; Leijon et al. 2011). This also showed that waterfowls which clinically healthy play as an evolution place of AI virus and by time the virus become more pathogen (Henning et al. 2010; Hewajuli & Dharmayanti 2012).

The QRERRRKR amino acid sequence of 3 isolates of H5Nx resembled with the common pattern existed in duck, is in accoordance with Wibawa et al. (2012). In this research, the same pattern was found in commercial layer (A/Ck/Lyr.Gunungsindur/Prl/2013), commercial broiler (A/Ck/Cianjur/Prl/2013), and duck (A/Dk/Brebes/Prl/2013). It was assumed that the waterfowls also played as a source of transmission in those cases. However, those 3 isolates had phenotypic difference with another 6 isolates in case of the longer period was needed to cause embryo death and the lower HA titre. There was a possibility that the viruses were came from low pathogenic virus which had undergone mutation or reasortion, so that had amino acid pattern in the cleavage site of hemagglutinin gene resembled with the pattern of the pathogenic virus. Gohrnandt et al. (2011) explained that a HPAI strain may be an

acquisition of a non-pathogenic AI strain which had experienced a mutation and had cleavage site region that characterized pathogenic AI strain. In spite of it did not show clinical symptoms, the waterfowls could continuously excrete virus, so it could be potentially mode of spreading pathogenic virus to another birds (Li et al. 2011). Zhao et al. (2012) said despite an AI virus having cleavage site with the amino acid pattern characterized of high patogenicity AI virus, however phenotypically the virus could be characterized as low pathogenicity.

The fact that 3 isolates of H5Nx had phenotypic resembling low pathogenic AI virus but evidently having amino acid sequence of QRERRRKR which was characterized as high pathogenic virus, confirmed the assumption that AI viruses that had been isolated in Indonesia were pathogenic viruses, in line with WHO (2011) and Wibawa et al. (2012) which reported that AI viruses circulated in Indonesia since first report in 2003 were highly pathogenic avian influenza (HPAI) H5N1 subtype and that the prevalence of low pathogenic AI virus in Indonesian poultry had not been found yet.

Phylogenetic Analysis and Genetic Distance

The electrophoresis of the PCR product is shown in Figure 2 and Figure 3. Phylogenetic analysis and genetic distances which described in Figure 4 and Table 7 showed that AI isolates in this study were divided into 2 clusters. Isolate 1, 2, 4, 6, and 7 were in same cluster with AI isolated from chicken in Banten in 2008 (GenBank: GU183461), Legok 2003 (GenBank: GU052426.1), and AI Legok isolated by Prolab in 2008. The Legok 2003 was the isolate obtained from the first AI outbreak in Indonesia, so it was assumed that isolate 1, 2, 4, 6, and 7 were the descendant of the AI virus that caused the first outbreak and had not a meaningful genetic mutation. Genetic distance between the isolates ranged 0.042-0.081. Isolate 8 and 9 which were isolated from duck and isolate 3 and 5 which were isolated from layer and broiler chicken had genetic similarity with duck isolated from Tegal 2012 (GenBank: KC417274.1) and Blitar 2012 (GenBank: KC417277.1). Genetic distance between the isolates was 0.003-0.029. The genetic similarity between AI viruses isolated from chicken with those were isolated from waterfowls was suspected due to of the existence of waterfowls reared around the chicken farms resulting in AI transmission from the waterfowls to the chicken. Henning et al. (2010) said that waterfowls is the source of transmission of AI virus to another surrounding birds. Water as a living place of the waterfowls become the media and source of AI virus infection. The fact that waterfowls are the main source of AI virus infection makes the implementation of prevention and control programs of AI virus become more difficult to be conducted (Hewajuli & Dharmayanti 2012). The genetic distance between the 2 clusters in this study was 0.062-0.131, this showed that all of the isolates in the 2 clusters still had close relationship with the coefficient of phylogeny was <0.3 and homology was >97% (Wibawa et al. 2012).

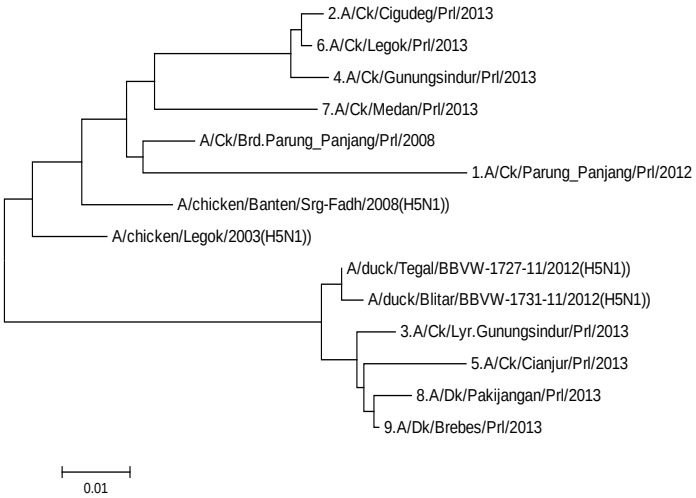


Figure 4. Phylogenetic tree of the hemagglutinin gene of AI viruses isolate 1-9 compare with AI virus from the previous outbreaks

Table 7. Genetic distances of the hemagglutinin gene between AI viruses used in the research with AI viruses from previous outbreaks

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
[1]															
[2]	0.025														
[3]	0.071	0.062													
[4]	0.074	0.065	0.003												
[5]	0.029	0.042	0.087	0.090											
[6]	0.066	0.080	0.129	0.131	0.055										
[7]	0.055	0.062	0.103	0.106	0.045	0.081									
[8]	0.085	0.076	0.013	0.016	0.080	0.125	0.103								
[9]	0.053	0.060	0.101	0.104	0.048	0.081	0.011	0.099							
[10]	0.096	0.083	0.025	0.029	0.086	0.127	0.106	0.022	0.108						
[11]	0.055	0.062	0.103	0.106	0.042	0.076	0.005	0.099	0.008	0.100					
[12]	0.049	0.059	0.107	0.110	0.038	0.071	0.043	0.103	0.053	0.108	0.045				
[13]	0.089	0.076	0.016	0.019	0.082	0.124	0.097	0.014	0.101	0.022	0.097	0.097			
[14]	0.085	0.073	0.013	0.016	0.078	0.120	0.093	0.008	0.095	0.017	0.093	0.097	0.006		

[1] #A/chicken/Banten/Srg-Fadh/2008(H5N1)); [2] #A/chicken/Legok/2003(H5N1)); [3] #A/duck/Tegal/BBVW-1727-11/2012(H5N1)); [4] #A/duck/Blitar/BBVW-1731-11/2012(H5N1)); [5] #A/Ck/Brd.Parung_Panjang/Prl/2008; [6] #1.A/Ck/Parung_Panjang/Prl/2012; [7] #2.A/Ck/Cigudeg/Prl/2013; [8]#3.A/Ck/Lyr.Gunungsindur/Prl/2013; [9] #4.A/Ck/Gunungsindur/Prl/2013; [10] #5.A/Ck/Cianjur/Prl/2013; [11] #6.A/Ck/Legok/Prl/2013; [12] #7.A/Ck/Medan/Prl/2013; [13] #8.A/Dk/Pakijangan/Prl/2013; [14] #9.A/Dk/Brebes/Prl/2013

CONCLUSION

All of the isolates used in this study were highly pathogenic avian influenza (HPAI). Six isolates were classified into H5N1 subtype, whereas the other 3 isolates were included into H5Nx subtype. Phylogenetic analysis and genetic distance between viruses showed that isolates in this study were divided into 2 clusters and still had close relationship.

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The Reliability of DIVA Test Based on M2e Peptide Exceed Those Based on HA2 or NS1 Peptides

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ABSTRAK

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Salah satu kelemahan utama vaksinasi pada avian influenza adalah bahwa vaksinasi tersebut tidak dapat memberikan perlindungan terhadap infeksi. Bila peternakan yang menerapkan vaksinasi terpapar virus dalam jumlah besar, infeksi subklinis dapat menyebar dalam peternakan tersebut dalam waktu yang lama tanpa diketahui. Kondisi tersebut hanya dapat dimonitor dengan alat tes DIVA (*differentiation of infected from vaccinated animals*), alat uji konvensional tidak dapat digunakan. Tes DIVA berdasarkan antibodi yang terbentuk akibat stimulasi virus bereplikasi merupakan tes DIVA yang paling sesuai. Untuk influenza H5N1 antibodi yang dimaksud antara lain antibodi terhadap M2e, protein NS1 dan peptida HA2 (HA_488-516). Tujuan penelitian ini adalah membandingkan level antibodi terhadap peptida M2e, NS1 dan HA2 pada ayam normal, vaksinasi dan infeksi (1, 2-3, ≥4 minggu pasca infeksi). Level antibodi diukur dengan ELISA menggunakan sintetik peptida sebagai antigen koting. Peptida yang digunakan antara lain: 4 buah peptida NS1 yang didasarkan pada berbagai lokasi pada protein NS1, peptida M2e dan HA2. Semua peptida dibiotinilasi pada ujung N nya. Koting peptida pada microtitre plate dilakukan secara langsung atau melalui jembatan streptavidin. Penelitian ini menunjukkan bahwa vaksinasi tidak merangsang pembentukan antibodi terhadap semua peptida. Ayam yang terinfeksi membentuk antibodi dengan level yang tinggi terhadap peptida M2e, tetapi sangat rendah terhadap peptida NS1 dan HA2. Antibodi terhadap peptida NS1 dan HA2 hanya dapat dideteksi dengan ELISA streptavidin-peptida. ELISA berbasis NS1 atau HA2 tidak dapat diandalkan sebagai tes DIVA untuk penyakit AI H5N1 pada ayam.

Kata Kunci: M2e, Protein NS1, Peptida HA2, Tes DIVA, H5N1

ABSTRACT

Tarigan S, Sumarningsih, Ignjatovic J. 2015. The reliability of DIVA test based on M2e peptide exceed those based on HA2 or NS1 peptides. JITV 20(2): 126-133. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1167

One of the most important disadvantage of vaccination against avian influenza is that it cannot protect vaccinated birds against infection. When vaccinated poultry are heavily exposed to the virus, prolonged, unrecognised, subclinical infection may persist on the farm. The condition can only be serologically monitored by a DIVA (differentiation of infected from vaccinated animals) test, whereas conventional diagnostic tests cannot be used. The DIVA tests based on an antibody response following virus replication is the most appropriate approach. For H5N1 influenza such antibodies includes those to the M2e and NS1 proteins and an epitope on the HA2 subunit (HA_488-516). The purpose of this study was to compare the magnitude of the antibody response in chickens vaccinated and infected with an H5N1 virus strain. For that purpose, sera collected from naïve, vaccinated and infected birds, at 1, 2-3, ≥4 weeks post challenge were used. Antibodies were measured by ELISA using biotinylated synthetic peptides as coating antigens. The peptides used include four NS1 peptides corresponding to different regions of the NS1 protein and HA_488-516and M2e peptides. Peptides were coated onto microtitre plates either directly or via a streptavidin bridge. The results showed that vaccination did not cause antibody conversion to any of the peptides, where as challenged birds developed a high antibody response to M2e but, low response to the NS1 and HA2 peptides. Antibodies to the later peptides were detected only by the streptavidin-peptide ELISA. The ELISA based on NS1 or HA_488-516 peptides, therefore, are not reliable for use as DIVA test in H5N1 avian influenza virus infection.

Key Words: M2e, NS1 Protein, HA2 Peptide, DIVA Test, H5N1

INTRODUCTION

One of the most important draw backs of avian influenza vaccination in poultry is the difficulty in disease surveillance. Vaccination provides protection against clinical disease and reduces the amount of virus shed by the infected birds, but it cannot provide complete protection against virus infection (Savill et al.

2006). When a vaccinated flock is exposed to HPAI virus challenge, it may result in subclinical infection that continues to circulate in the flock unrecognized by the farmers. This situation is compounded by the fact that a practical diagnostic tool is not available that can be used to detect the presence of subclinical infection. The common serological test such as HI test, ELISA or Agar gel precipitation test could not be used, as they are unable to differentiate between antibodies in birds due to infection and those due to vaccination. Virus isolation, PCR or rapid antigen detection test are either too expensive or insensitive.

Serological test that can identify infected among vaccinated birds are termed DIVA (Differentiation of Infected from Vaccinated Animals) test. Different DIVA strategies for avian influenza have been reviewed previously (Suarez 2005; Tarigan 2015). DIVA strategy based on heterologous-neuraminidase was successfully applied in Italy (Capua et al. 2003; Capua et al. 2004). The prerequisite was that the vaccine used had to be generated from virus subtypes with neuraminidase heterologous to that of circulating virus, preventing the application of this strategy in other countries, including Indonesia. In addition, the indirect iFAT (fluorescent antibody test) used to identify infected birds in this strategy is also not practical.

Unlike DIVA test based on heterologous-neuraminidase, DIVA test based on the external domain of M2 protein (M2e) is independent from the vaccine subtype since antibodies to M2e develop only in infected animals but not in vaccinated with killed AIV vaccines. ELISA based on either synthetic M2e peptide or bacterial expressed M2e protein has been used to measure the M2e antibody response (Lambrecht et al. 2007; Kim et al. 2010; Hemmatzadeh et al. 2013; Hadifar et al. 2014; Tarigan et al. 2015). The M2e-ELISA was reported to have high specificity but rather low sensitivity. The low sensitivity is attributed to the fact that M2e antibody can be detected in birds not sooner than 7 days of infection, and also some birds fail to seroconvert to M2e. Additional DIVA test capable of detecting infection in those M2e-negative, but infected birds, would be advantageous in order to increase the sensitivity of DIVA testing. The purpose of this study was to investigate the possibility of an ELISA test based on the influenza virus nonstructural-1 (NS1) protein and also on the recently identified epitope in the HA2 domain of haemagglutinin H5 (HA_488-516) as possible supplements to the M2e DIVA test (Khurana et al. 2011). This possibility was investigated by comparing the magnitude of the antibodies to those proteins or peptides in naïve, vaccinated and infected chickens.

The NS1 protein is the only true non-structural protein in Influenza viruses and interest in developing DIVA tests based on this protein have been considered. However, the accuracy of test based on this protein as DIVA test is still contradictory between different studies. Some studies found that the NS1 based tests are sensitive and specific as DIVA test (Birch-Machin et al. 1997; Golchinfar et al. 2014; Ozaki et al. 2001; Takeyama et al. 2011), while others have found the reverse (Avellaneda et al. 2010). In this study, the accuracy of NS1-based ELISA as DIVA test was evaluated thoroughly by analyzing different fragments of the protein and different condition of ELISA. In addition, antibody to an epitope in the HA2 domain of haemagglutinin H5 (HA_488-516) suggested as being a reliable marker for H5N1 infection in human, has yet to confirmed in poultry (Khurana et al. 2011).

MATERIALS AND METHODS

Peptide

Four NS1 peptides, one HA peptide, and M2e peptide were synthesized by VCPBIO Inc. China. Following are the location and amino acid sequences of the peptides NS1_34-49 (Biotin-DRLRRDQKSLR GRGNT), NS1_23-42 (Biotin-ADQELGDAPFL DRLRRDQKS), NS1_87-98 (Biotin-TDMTLEEM SRDW), NS1_221-233 (Biotin-QKRKMARTIESEV), HA_488-516 (Biotin-DYPQYSEEARLKREEISG VKLESIGIYQI), M2e (Biotin-MSLLTEVETPTR NEWECRCSDSSD). All peptides, which were biotinylated at the N-terminal, had at least 90% purity.

Serum

Sera used in this study were collected from chicken experimentally infected with H5N1 influenza virus (A/Chicken/WestJava/Sbg-29/2007). The challenge experiment had been described in our previous study (Tarigan et al. 2015). Briefly, 200 layer chicken were divided into 4 groups (A, B, D and C). Groups A, B, and D were vaccinated with a commercial-killed-H5N1 vaccine 3, 2 and 1 times, respectively. Group C birds were not vaccinated and served as a control. Two weeks after the last vaccination, randomly selected birds from each groups including the control were challenged with an isolate of H5N1 virus. For the current experiment, 18 sera from each group of pre-vaccination, post vaccination, 1, 2-3, and ≥4 weeks post challenge were selected.

Direct-peptided- and streptavidin-peptided-coated ELISA

Two types of ELISA were used based on the method used for coating the peptides on the 96-well microtitre plate. In the first ELISA, each biotinylated peptide was disolved in carbonate buffer (pH 9.6) at 5

µg/ml then added to the plates at 100µl/well). After leaving at 4°C overnight, the plate was blocked with 5 mg/ml of non-fat-skim milk in PBS (pH 7.2), 150 µl/well for 2 hours. Serum diluted at 1:100 in PBST(PBS, 0.5% Tween 20) supplemented with 5% normal rabbit serum was added at 100 µl/well, then incubated at 37°C for 2 hours. In each plate, negative control SPF chicken serum was added. After washing four times with PBST, HRP-rabbit-anti-chicken IgG conjugate (Sigma Co.) diluted 1:5000 in PBST supplemented with 5% normal rabbit serum was added, then incubated at 37°C for 2 hours. After washing four times with PBST, solution of substrate and chromogenic ABTS was added and the absorbance (A_{420}) measured with a spectrophotometer microtitre plate reader.

In the second type of ELISA, streptavidin disolved in carbonate buffer at 4 µg/ml, was added 100 µl/well, and left overnght at 4°C. Streptavidin used in this study was produced in the previous study (Tarigan & Sumarningsih 2014). After washing four times with PBST, biotinylated peptide diluted in PBS at 5 µg/ml was added at 100 µl/well. The plate was blocked with skim milk and the remining procedures were the same as in the first type of ELISA.

A preliminary assay was carried out to prove that streptavidin coated to the plates did specifically bind to the biotin, and to determine the optimum concentration of streptavidin for the assay. Streptavidin was diluted to 4, 2, 1, 0.5, 0µg/ml in carbonate buffer and added to mocrotitre plate at 100 µl/well. After leaving at 4°C overnight, the plate was blocked with skim milk for 2 hours. Biotynilated chicken IgY, produced in the previus study (Tarigan et al. 2015), was added at 1:1600 and 1:3200 dilution in PBS and incubated at 37°C for 2 hours. After washing four times with PBST, HRP-anti chicken IgG (Sigma Co.) at 1: 5000 dilution was added and incubated at 37°C for 2 hours. After washing four times with PBST, solution of substrate and chromogenic ABTS was added and the absorbance (A_{420}) measured with a spectrophotometer microtitre plate reader.

Statistical analysis

The level of antibody, indicated by ELISA's OD, to each peptide in each group of bird (pre-vaccination, post-vaccination, 1-week-post challenge, 2-3-week-post challenge, and ≥4-week-post challenge) was presented as the mean and 95% confidence interval of the mean of the ELISA's OD. Differences between groups were analised by one-way ANOVA (analysis of variance). All statistical analyses were carried out using a commercial statistical package (IBM® SPSS® Statistics).

RESULTS AND DISCUSSION

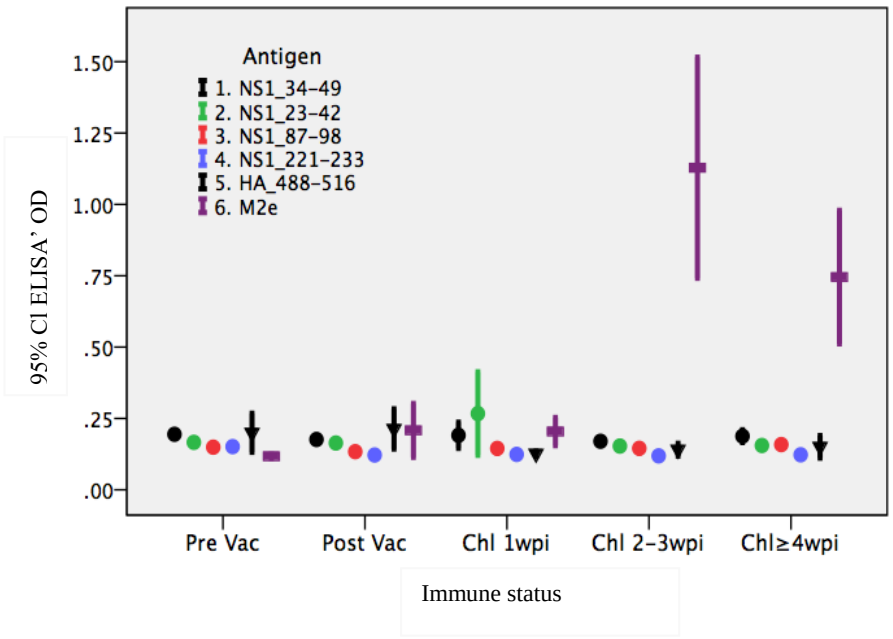
Results

Antibodies to NS1, HA_488-516 and M2e, measured by direct peptide ELISA in chickens of various immune or infection status, are presented in Figure 1. No antibody to any of the four NS1 peptides, the HA_488-516 peptide or M2e peptide was detected in chickens before and after vaccination. Antibody to M2e rose markedly after 1 week and remained high for several weeks after challenge. Unlike antibody to M2e, no antibody conversion was observed to any of the NS1 or HA_488-516 peptides.

There are two possibilities regarding the lack of measurable antibody conversion to the NS1 and HA2 peptides. The first possibility was that antibodies did not develop in those infected birds. This possibility seemed unlikely because antibody to NS1 should be detected whenever replication of influenza virus take place in immuno-competent animals. The strong antibody response to M2e indicated that a substantial replication of H5N1 virus must have had taken place in those infected birds. The second possibility was that the direct peptide ELISA used in this study was not sensitive enough to detect the presence of NS1 or HA_488-516 antibodies. Onepossible cause of this insensitivity was that the NS1 and HA2 peptides did not bind to the plates, or were unable to bind the antibodies once the peptides were immobilised on the plates. This problem was aliviated by the use of the streptavidin-peptide ELISA.

When added to the streptavidin-coated, protein-blocked plates, biotinyl-IgY was bound to the plate efficiently. In contrast, no biotinyl-IgY was retained when added to a non-streptavidin-coated, protein-blocked plates (Figure 2). This results indicated that the binding activity of streptavidin coating was specific and efficient. The concentration of streptavidin on coated plates that maximally bound the biotinyl IgY was around 4 µg/ml, and this concentration was used in all assays in this study. When the streptavidin-peptide ELISA was used to assay the chicken sera, the results, regarding antibody to M2e, were comparable to the direct peptide ELISA described above.

This streptavidin-peptide ELISA, however, was able to show that the level of antibody to all peptides in the sera of infected birds were higher than those in the non-infected birds (Figure 3). Although the increase of antibodies were only slight, they were statistically significant ($P>0.05$). The statistically significant increases or differences were between pre-vaccinated and challenged (≥4 wpi), and post-vaccinated and challenged (≥4 wpi) sera, to all peptides (Table 1).



Pre Vac = Pre vaccination;
Post Vac = 2 week post vaccination (1, 2 and 3 times vaccination);
Chl 1 wpi = 1 week post challenge;
Chl 2-3 wpi = 2 and 3 weeks post challenge;
Chl ≥ 4wpi = 4 week or longer after infection or challenge

Figure 1. Antibody conversions to four NS1, HA_488-516 and M2e peptides in chickens after vaccination and challenge with H5N1 virus

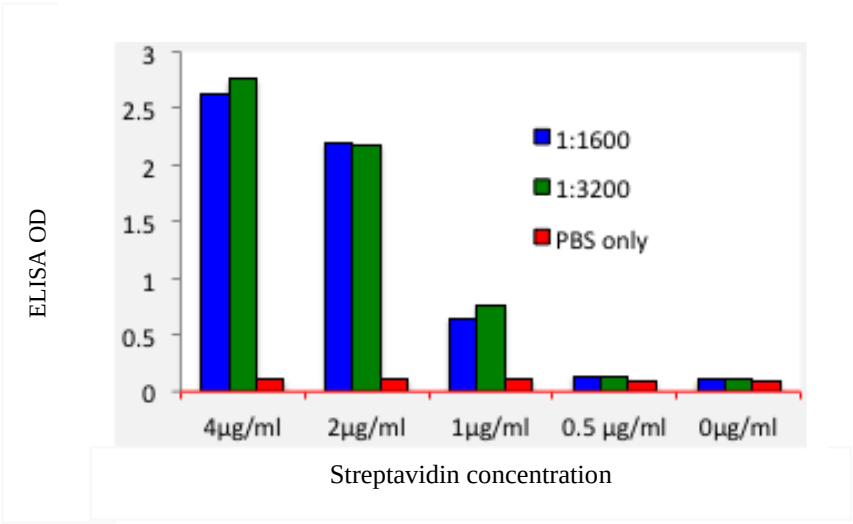
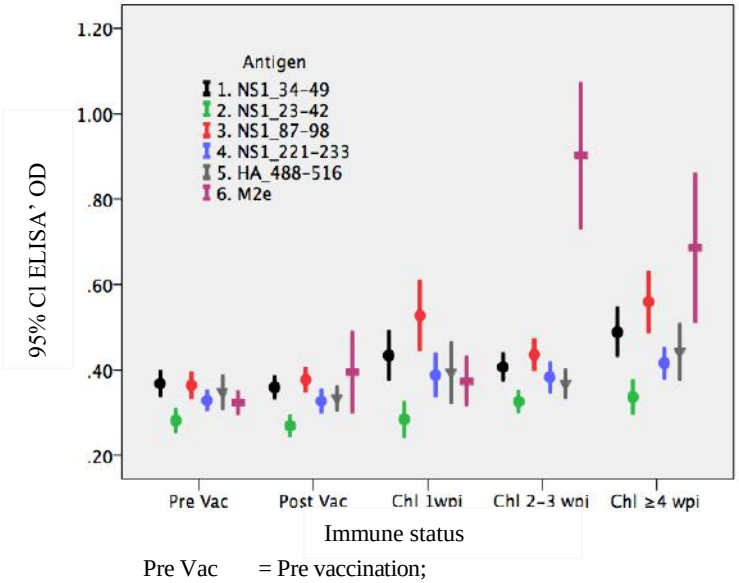


Figure 2. Binding of biotinylated IgY to streptavidin coated onto wells of microtitre plate. Biotinylated IgY was added to streptavidin-coated, protein-blocked microtitreplate. The biotinylated IgY bound to streptavidin was detected by HRP-anti-chicken IgG and the quantity of biotinylated IgY bound to the strepatvidin was correlated with the ELISA's OD



Pre Vac = Pre vaccination;
Post Vac = 2 week post vaccination (1, 2 and 3 times vaccination);
Chl 1 wpc = 1 week post challenge;
Chl 2-3 wpc= 2 and 3 weeks post challenge;
Chl ≥4 wpc = Post challenge 4 week or longer

Figure 3. Antibody conversions to NS1, HA2 and M2e peptides in chicken after vaccination and challenge with H5N1 virus

Table 1. Antibody to NS1 (NS1_23-42, NS1_34-49, NS1_87-98, NS1_221-233), HA2 and M2e peptides in chickens that increased significantly (P<0.05) after challenge with H5N1 ELISA streptavidin

		1 week post challenge	2 and 3 weeks post challenge	Post challenge 4 week or longer
NS1_23-42	Pre vaccination		✓	✓
	2 week post vaccination (1, 2 and 3 times vaccination)		✓	✓
	1 week post challenge			
	2 and 3 weeks post challenge			
NS1_34-49	Pre vaccination			✓
	2 week post vaccination (1, 2 and 3 times vaccination)			✓
	1 week post challenge			
	2 and 3 weeks post challenge			
NS1_87-98	Pre vaccination	✓	✓	✓
	2 week post vaccination (1, 2 and 3 times vaccination)	✓		✓
	1 week post challenge			
	2 and 3 weeks post challenge			✓
NS1_221-233	Pre vaccination		✓	✓
	2 week post vaccination (1, 2 and 3 times vaccination)			✓
	1 week post challenge			
	2 and 3 weeks post challenge			
HA_488-516	Pre vaccination			✓
	2 week post vaccination (1, 2 and 3 times vaccination)			✓
	1 week post challenge			
	2 and 3 weeks post challenge			
M2e	Pre vaccination		✓	✓
	2 week post vaccination (1, 2 and 3 times vaccination)		✓	✓
	1 week post challenge		✓	✓
	2 and 3 weeks post challenge			

✓ = Antibody (ELISA OD) was significantly increased (P<0.05)

No differences in all antibodies between pre and post-vaccinated sera. The level of antibody to NS1_87-98 peptide was significantly higher in the 1-week-post-challenge sera than in pre- and post-vaccinated sera. The higher level at the 1-week-post-challenge sera was not observed in antibody to other peptides, even that to M2e.

Discussions

This study shows that M2e-based ELISA is superior to NS1- or HA2-based ELISA as DIVA test. As far as we are aware, no study has been carried out previously comparing M2e ELISA and NS1-peptide-based ELISA as DIVA test.

Influenza-NS1 protein consists of 230 amino acids and two functional domains: the RNA-binding (aa residues 1-73) and the effector domain (aa residues 74-230). The RNA binding domain funtions to inhibit action of interferon (Donelan et al. 2003; Min & Krug 2006). The effector domain functions to enhance viral mRNA translation, deregulate cellular mRNA processing, inhibit dsRNA-activated protein kinase and activate phosphoinositide 3-kinase (PI3K) signaling (Hale et al. 2008).

The NS1 protein is the only true non-structural protein in influenza viruses (Donelan et al. 2003). Being the non-structural protein, it is expressed only during viral replication inside the host cells but the protein is not packed into the virion. Driven by the assumption that animals vaccinated with killed AIV vaccines do not develop specific antibodies to the NS1 protein, as do animals infected by life virus, interest in developing a DIVA test based on the NS1 protein has been long present. However, there is still disagreement between different studies on the accuracy of NS1-based ELISA used as DIVA test. Most studies showed that NS1-based tests had low accuracy as a DIVA test, as was found in this present studies (Tumpey et al. 2005; Dundon & Capua 2009; Avellaneda et al. 2010). Other studies, however, indicated that NS1-based ELISA ispotentially useful as DIVA test (Birch-Machin et al. 1997; Ozaki et al. 2001; Tumpey et al. 2005; Takeyama et al. 2011; Wang et al. 2011; Golchinfar et al. 2014). In spite of that potential of NS1-based ELISA as DIVA test, the test had rather low specificity because some vaccinated animals were also seropositive although with low antibody titres. The NS1 antibodies are presumed to be induced by the NS1 protein derived from cellular debris of chicken embryo contaminating the vaccines (Tumpey et al. 2005). The NS1 ELISA has not only low specificity but also low sensitivity. The low sensitivity is associated with the nature of the protein that is poorly immunogenic. The NS1 antibody is usually in low titre and rapidly disappears (Tumpey et al. 2005; Avellaneda et al. 2010). Immune response to NS1 may also be species-dependent. An experimental study reported that infection of chicken with a LPAI isolate caused antibody conversion to NS1 protein only in 3 of 14 birds, and the antibody was detected only at day-3 post infection. Infection of turkey with the same isolate produced higher proportion of seroconversion and the antibody was detected at day-5 to day-10 post infection (Dundon & Capua 2009).

There are several possible causes of the NS1-antibody undetectable in infected chicken. The first possible cause is that the assay used is not sensitive enough to detect the presence of the antibodies. In this present study, four synthetic peptides with amino acid sequence based on the regions of the protein considered to be immunogenic based on hydrophobic analysis. A peptide comparable to that NS1_34-49 in this study has previously been demonstrated to be sensitive when used as coating in ELISA in detecting NS1 antibody (Tumpey et al. 2005). Test based on peptide NS1_23-42 that proceeds at the N-terminal, and overlaps 9 amino acids with NS1_34-49 also failed to detect the presence of NS1 antibody. As a matter of fact, Tumpey et al. 2005 shows that the peptide is more specific, although less sensitive, than the recombinant–whole-NS1 protein. This finding lend support to the opinion that ELISA based on the whole-NS1 protein is not necessary more accurate than that bases on NS1-synthetic peptide in detecting the antibody to the NS1 protein.

The C terminal end of NS1 protein that contain the PDZ-ligand binding motif (PBM), ESEV, has been proven to be immunogenic, and animals infected by influenza viruses become seroconverted to synthetic peptides which sequence is based on the C terminal part of the NS1 protein (Birch-Machin et al. 1997; Dundon et al. 2006). In this present study, however, the sera from infected birds did not contain antibody to a comparable peptide, NS1_221-233, assayed with the direct-peptide coating ELISA.

Sera from human survived from H5N1-virus infection have been shown to contain antibody to a HA2 peptide, HA_488-516 (Khurana et al. 2011). The antibody reported to be specific for H5N1-virus infection as it was not detected in sera from human vaccinated or infected with seasonal influenza virus, or vaccinated with subunit H5N1 vaccine (Khurana et al. 2011). These results suggest that the peptide deem a good candidate for DIVA test in poultry. However, our study showed that the infected birds failed to be positively converted to the same HA_488-516 peptide.

It is not only the peptides themselves, the ELISA format, the direct coatingof peptides onto the wells of ELISA plates, used in this study was also similar to the previous studies (Tumpey et al. 2005; Dundon et al. 2006). Therefore, the probability that the peptides were notimmobilised on the microtitre plate as the cause of the failure to detect the NS1 antibody in sera of

challenged birds in our study seems unlikely. This possibility was partially ruled out by the streptavidin-peptide ELISA. The streptavidin coated onto the plates has been shown to specifically bind to biotinyl substances. Since the peptides used were all biotinylated at the N terminal, the peptide were expected tobind to the plate only through its N-terminal 'tip'; consequently, if asample serum contains NS1 antibody, it should react with the immobilised peptide efficiently. The fact that the ELISA's ODs of challenged sera increased significantly as compared to those before challenge indicated that the streptavidin-peptide coated ELISA is more sensitive than the direct-peptide coated ELISA. Since the increased were only slight even in the improved assay, it indicates that the conversion of antibody to NS1 protein in the infected birds is only mild and only detected by a very sensitive assay.

In summary, this study shows that antibody to either M2e, HA_488-516, NS1 peptides, are absent in bird naïve or vaccinated with killed H5N1 vaccines. Antibody conversion to M2e is more consistent and its magnitude is much higher than that to HA_488-516 and peptide NS1 peptides. Consequently, M2e-based is much more reliable than NS1- or HA2-based ELISA as DIVA test. Antibody conversion to HA_488-516 and peptide NS1 in infected chickens is extremely mild and therefore difficult to detect.

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Newcastle Disease Virus Infection Study on Duck and Chicken in Subang District

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ABSTRAK

Panus A, Setiyaningsih S, Mayasari NLPI. 2015. Kajian infeksi virus *Newcastle Disease* pada bebek dan ayam di Kabupaten Subang. JITV 20(2): 134-147. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1168

Tujuan dari penelitian ini adalah untuk mendeteksi dan mengetahui keragaman antigenik virus Newcastle disease (NDV) yang bersirkulasi di Kabupaten Subang. Sampel usapan kloaka, usapan orofaring dan serum diambil dari 393 ekor ayam dan 149 bebek dari penampungan, peternakan dan pasar unggas di 10 kecamatan di Kabupaten Subang. Screening NDV pada sampel pool (5-7 individu per pool) dengan real-time Reverse-Transcription Polymerase Chain Reaction (rRT-PCR) matrix (M) menunjukkan 19 dari 67 pool kloaka (28,3%) dan 8 dari 67 pool orofaring (11,9%) ayam terdeteksi NDV; 18 pool dari 67 pool ayam (26,9%) menunjukkan virus diekskresikan melalui kloaka dan orofaring. Sementara pada sampel itik, NDV terdeteksi hanya pada kloaka yaitu 8 dari 30 pool (26,7%). Total 18 isolat berhasil diisolasi dari sampel usapan kloaka dan usapan orofaring individu yang menunjukkan karakter antigenik yang homogen, namun beberapa isolat menunjukkan variasi dengan titre sampai 2 Log2 menggunakan antisera LaSota dan 2-4 Log2 dengan antisera Komarov. Mayoritas isolat menunjukkan afinitas lebih tinggi terhadap antisera Komarov, yang mengindikasikan semua isolat adalah NDV galur ganas. Karakterisasi patogenisitas dengan uji elusi hasilnya menunjukkan 3 isolat masuk ke kelompok galur mesogenik dan 15 isolat ke kelompok galur velogenik, sedangkan dengan rRT-PCR fusion (F) menunjukkan 100% isolat merupakan galur ganas (mesogenik/velogenik). Deteksi antibodi spesifik terhadap NDV pada 408 serum dengan uji HI menunjukkan 48 serum (12%) positif dengan kisaran titre 1 sampai 8 Log2; hanya sekitar 13% ayam yang divaksin menunjukkan titre protektif (≥3 Log2). *Newcastle disease* masih endemik di Kabupaten Subang dengan variasi antigenik galur virus yang bersirkulasi relatif tidak telalu bervariasi.

Kata Kunci: Newcastle Disease, rRT-PCR, Virulensi, Keragaman Antigenik, Antibodi

ABSTRACT

Panus A, Setiyaningsih S, Mayasari NLPI. 2015. Newcastle Disease Virus infection study on duck and chicken in Subang district. JITV 20(2): 56-69 DOI:http://dx.doi.org/10.14334/jitv.v20i2.1168

The objectives of this research were to study Newcastle Disease Virus (NDV) infection in Subang area and to examine the diversity of the circulating NDV. Swabs of cloacal and oropharynx, and serum were sampled from total of 393 chickens and 149 ducks in backyard farms and live bird markets located in 10 subdistricts. Screening of NDV in pool of 5-7 samples by real-time Reverse-Transcription Polymerase Chain Reaction (rRT-PCR) matrix (M) showed 19/67 (28.3%) cloacal and 8/67 (11.9%) pharyngeal pools of chicken samples; 18/67 (26.9%) of the pools excreted virus via cloaca and oropharynx, while the duck pools of 8/30 (26.7%) shed virus from cloaca. Virus isolation attempted on individual sample from positive pools yielded 18 isolates which the majority of the isolates showed homogeneous antigenic character, only some of these showed variations up to 2 Log2 with Lasota and 4 Log2 with Komarov antisera. Majority of isolates had a higher affinity to Komarov indicating their propencity to virulent strains. Pathogenicity examination using elution test showed 3 isolates virus were grouped to mesogenic strains and 15 isolates to velogenic strain, in agreement with rRT-PCR fusion results. HI test on 408 sera showed that NDV antibody was detected in 48 (12%) birds with titres ranging from 1 to 8 Log2; only about 13% of vaccinated chickens demonstrated protective antibody titre (≥3 Log2). Newcastle disease is still endemic in Subang with relatively low antigenic variation among circulating strains.

Key Words: Newcastle Disease, rRT-PCR Detection, Detection of Virulence, Antigenic Diversity, Antibody

INTRODUCTION

Newcastle Disease (ND) is one of serious diseases in poultry because it is very contagious, spread rapidly and attack some species of birds at all age. Mostly this outbreak attacks intensive poultry as follows: chicken, turkey, duck, quail, and pigeons. ND spread all over the world and potentially causes economy losses in poultry industry. In addition of poultry, this disease infect and causes death in wild birds as well. ND cases were firstly found and reported in the mid of 1920 in Indonesia (Java Island) and England (OIE 2012), then spreading in a few years later and becoming endemic in many countries (Ashraf & Shah 2014). Nowadays, almost all regions in Indonesia are affected and no one area or island is free from ND. In spite of mortality rate caused by ND was controlable, the effect in production is still a problem. Moreover, the impact of other losses is the costs for controlling the disease and also stopping export from ND endemic countries (Brown et al. 1999).

The outbreak caused by ND can be acute or chronic and infecting all species of birds especially chicken, both domestic and purebred. The outbreak occured in the field may caused by various strain of ND virus. According to the severity-level of the outbreak in chicken, Newcastle Disease Virus (NDV) was classified into three pathotypes namely lentogenic, mesogenic, and velogenic. Velogenic strain is distinguished into neurotropic and viscerotropic form (Aldous & Alexander 2001).

The loss caused by ND are morbidity and mortality which in infected poultry the rate may reach 100% caused by velogenic strain especially in sensitive chicken groups and under 10% in mesogenic strain (OIE 2008). In the developing countries where the livestock industry is growing very rapidly, the losses affected by NDV outbreak are not only mortality but also expenditure additionaly cost used for vaccination, biosecurity and depopulation. Even the free ND countries, have to spend on periodic testing in order to maintain free status from ND which needed for trading license. Moreover, in the developing countries as endemic ND, the impacts are not only economy losses but also affecting health and socioeconomic condition of lower-class society, whose quality and quantity of eggs and meat consumed decreased caused by ND (Alexander & Senne 2008). In 2002, ND outbreak in California, United States caused losses 200.000.000 US$ as an impact of depopulation (Kapczynski & King 2005). The losses affected by ND in layer are mortality and reduction of egg production, while causing growth disorder and reduction of body weight in boiler. Data of OIE (2009) showed in 2007, about 1500-8000 chickens were infected by ND every month in Indonesia. Moreover, according to Xiao (2012) in 2009 and 2010, ND outbreak occured in comercial chicken in Indonesia causing 70-80% mortality. ND is still become a major problem in the poultry industry despite the vaccination carried out routinely (Samal 2011). Therefore, ND is a serious threat for poultry in Indonesia. Subang area in West Java is one of buffer zones of poultry production, particularly for broilers and layers. Totally 44.049.739 poultry population was reported in 2013 (DISNAK 2013). Newcastle Disease is endemic in Indonesia including in Subang area. DISNAK (2013) recorded, there were 258 birds suddently died caused of ND infection in 2010 and it was confirmed by rRT-PCR using cloacal and oropharyngeal swabs and organ samples. Mass dead might occur if it was not handled properly. Annually survey by Balai Penyidikan dan Pengujian Veteriner (BPPV) Subang in unvaccinated ND backyard birds in 2011, found 10 out of 131 serums tested were positive of ND with titre range 2-5 Log2 (BPPV 2011). In 2012, 12 out of 37 serums tested were positive of ND with titre range 1-4 Log2 (BPPV 2012), and in 2013, 184 out of 359 serums tested were positive of ND with titre range 2-8 Log2 (BPPV 2013). These results show that ND is still endemic in Subang area. As the basis of consideration for efective control measures and prevention, it is neccesary to conduct NDV isolation and detection of antibody against ND in ducks and chickens in Subang area.

For the time being, investigation of ND in Subang area is still limited. Commonly, the diagnosis was based on clinical symptoms, pathological alteration and serological test. Therefore, diagnostic technique with high sensitivity to detect and confirm NDV infection in ducks and chickens in Subang area is required.

MATERIALS AND METHODS

Samples

Samples were taken from 10 subdistricts in Subang, that were Binong, Ciasem, Cipendeuy, Cipunagara, Compreng, Pagaden, Pusaka Nagara, Subang, Sukasari and Tambak Dahan. These areas were selected because population of fowls were centralized in those locations (market, shelter, farm) and endemic area of ND as well.

Standard antigen and antisera and Kit

ND virus standard (4HAU) LaSota strain (collection of FKH IPB), specific standard antisera against LaSota and Komarov strain were used for HI test (collection of BBPMSOH). QIAamp® Viral RNA Mini Kit (Qiagen) was used for RNA virus extraction. Ag-Path ID™ One-Step RT-PCR kit from *Life Technologies* with 96 optical plates in *Applied Biosystems 7500 Real Time PCR System Software Version* 1.4.0 were used for Real time RT-PCR.

Collection of swabs and serum samples

Swabs of cloacal and oropharynx were taken from chickens and ducks from the bird's shelter, livebirds market and poultry farms in 10 areas in Subang using sterile cotton swabs inserted in microtube 2 ml contains *Brain Heart Infusion Broth* (BHIB). The temperature was kept cool (4-8°C) until arrival in the laboratorium.

Pooling swabs of cloacal and oropharynx samples consist of 5-7 individual samples in each pool was based on swab types, birds, location and time of sampling. The sample pool was subsequently used for rRT-PCR test using *primer matrix* (M). Blood was collected via branchial vein from each individu along with swab samples.

real time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) Test

To detect the presence of genetic material of NDV extracted from swabs of cloacal and oropharynx, rRT-PCR test with NVSL protocol (2005) was done. RNA virus isolation was extracted based on *QIAamp® Viral RNA Mini Kit* (Qiagen) standard procedure. rRT-PCR amplification using *Ag-Path ID™ One-Step RT-PCR kit* from *Life Technologies* in *Applied Biosystems 7500 Real Time PCR System* was conducted. Cycles of rRT-PCR was performed in 45°C for 10 minutes, 95°C for 10 minutes, 95°C for 10 minutes, 56°C for 32 seconds, and 72°C for 10 seconds. The result was analyzed by *Applied Biosystems 7500 Real time* PCR *SystemSoftware Version* 1.4.0. Primer and probe were used are presented in Table 1.

Virus isolation in SPF embryonated chicken egg

Swabs of cloacal and oropharynx samples used as inoculum were from individual bird sample from positive rRT-PCR M pool. As much as 0.2 ml inoculum containing *penicillin-streptomycin* (9:1) and incubated for 30 minutes at ambient temperature (25-27°C) was injected in allantoic cavity of Specific Pathogen Free (SPF) embryonated chicken egg. Eggs were incubated in incubator at 37°C for 4-7 days and observed 3 times a day to check the viability of embryo (OIE 2012). Isolates obtained from alantoic liquid were reconfirmed with rRT-PCR matrix (M).

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Test

Procedures of HA and HI test was done by micro methods (OIE 2012), performed by adding 25 µl 0.85% *phosphate buffered saline* (PBS) into micro plate in 1st-12th pit using micro pipet. In the 1st pit 25 µl serum standard was added and diluted, than moved into 2nd-11th pit. A total of 25 µl 4HAU ND virus suspension was added into each 1st-10th and 12th pit, and then incubated at ambient temperatute for 15 minutes and 25 µl suspension of 1% red blood cells (rbc) was added into 1st-12th pit, homogenized and incubated at ambient temperature (25-27°C) for 40 minutes. Positive result marked by occurance of resistance hemagglutination in form of precipitation of rbc on the bottom of micro plate pit. Titre of HI was determined based on the highest serum dilution that was still showed precipitation (agglutination inhibition). HA and HI test were performed 3 times.

Elution-time test

Test was performed based on Ezeibe & Ndip (2005) procedures. A total of 50 µl PBS solution was put into micro plate pit, then 50 µl virus suspension was added into 1st pit, and diluted into 1st-10th pit. As much as 50 µl PBS was added into each 1st-12nd pit, followed by 50 µl suspension of 0.6% rbc into 1st-12nd pit, homogenized and incubated at ambient temperature for

Table 1. Primers and Probes used for rRT-PCR matrix (M) and fusion (F)

Target of gene	Primer/genom probe targets	Sequence(5'→3')	Reference
APMV-1 M (matrix)	M+4100 forward	AGTGATGTGCTCGGACCTTC	Wise et al. (2004)
	M+4169 probe matrix	[FAM]TTCTCTAGCAGTGGG ACA GCC TGC[TAMRA]	
	M-4220 reverse	CCT GAG GAG AGG CAT TTG CTA	
APMV-1	F+4829 forward	GGTGAGTCTATCCGGARGATACAAG	CVL (2007)
F (Fusion)	F+4939 reverse	AGCTGTTGCAACCCCAAG	
	F+4894 Probe 1 (Virulent)	[FAM]AAGCGTTTCTGTCTCCTTCCTCCA [TAMRA]	

40 minutes, then hemagglutination was observed. Elution time was determined based on time of complete hemagglutination was observed on highest dilution until the precipitation of rbc showed (elution). Elution-time test was performed 3 times.

Data analysis

Data were analyzed descriptively and statistically to determine standard mean deviation (SD). The average of antibody titre was calculated by geometric mean titre (GMT) by the formula:

$$\text{Log2 GMT} = \frac{(\text{Log2 t1})(\text{S1}) + (\text{Log2 t1})(\text{S1}) + \ldots + (\text{Log2tn})(\text{Sn})}{\text{N}}$$

Information:
- N = Number of observed serum
- T = Antibody titre at the highest dilution (which was still may inhibit agglutination of red blood cell)
- S = Number of titrated serum
- n = Antibody titre of the n^{th} sample

Coefficient of variation/CV from immune response was expressed by following formula:

$$CV = \frac{S}{\bar{X}} \times 100\%$$

Description:
- CV = Coefficient of Variant,
- S = Standard Deviation,
- $\bar{X}$ = Average of antibody titre

RESULT AND DISCUSSION

Detection of NDV in pool of swabs of cloacal and oropharynx with rRT-PCR matrix (M)

There were 542 samples succesfully collected consist of 149 ducks and 393 chickens sample comprised of 108 broilers, 148 broiler parent stocks, 15 layers and 122 lokal chickens from 10 areas in Subang district.

Testing for 97 pools swabs of cloacal resulted in 27 pools (29%) of positive which spreaded in 9 areas, 8 pools (7%) oropharynx swabs positive spreaded in 3 areas and 18 pools (18%) of cloacal and oropharynx swabs positive spreaded in 6 areas while none was positive from Cipendeuy area (Table 2). ND virus was only detected in cloacal swabs of ducks (8/30 pools), 19 pools cloacal swabs, 8 pools oropharynx swabs and 18 pools cloacal and oropharynx swabs (Table 1). Ducks tend to excrete the virus via cloaca according to the findings reported by Saepulloh & Darminto (2005) which were 14 (13%) isolates from cloacal and none from oropharynx swabs of 106 ducks in Kalimantan.

The highest number of positive M cloacal swabs pools (7) were obtained from Tambak Dahan area. The highest number of positive M oropharynx swabs pools (4) were obtained from Cipunagara area and the highest number of positive M cloacal and oropharynx swab pools (8) were obtained from Binong area, while in Cipendeuy area in pool of cloacal or oropharynx swabs, NDV were not detected.

Isolation of NDV in SPF embryonated chicken eggs

NDV virulence could be determined based on the infected embryo's death time. According to Cattoli et al. (2011), NDV causing embryo's death in more than 90 hours after inoculation was grouped in to lentogenyc strain, and between 60-90 hours grouped in to mesogenyc strain, while less than 60 hours, was grouped into velogenyc strain.

Inoculation of 128 cloacal swabs positive rRT-PCR M, 10 isolates were obtained, and from 76 positive oropharynx swabs, 8 isolates obtained. Totally, 18 NDV were succsessfully isolated and they were 3 isolates from 3 ducks and 15 isolates from chickens. The virus excreted via chicken's cloaca and oropharynx was balanced; 7 isolates from native chicken cloaca and oropharynx, 6 isolates from broiler oropharynx and 2 isolates from 2 native chicken oropharynx. Four out of 18 isolates were excreted from cloacal and oropharynx from 2 native chickens. In Binong area, pool of cloacal and oropharynx swabs sample were not contain NDV (negative rRT-PCR M) so isolates were not obtained from layer (Table 1). Transmission route of virus from the host body affected by tropism tissue of NDV. The virus which is replicated in respiratory tract will be shed through mouth and nostril and NDV replicated in digestive tract will be shed through cloaca. During incubation, virus replicated at the entry site. Virulent NDV strain (mesogenyc and velogenyc) could invaded into blood vessels, following the blood circulation and replicated in visceral organs, then excreted through the feces (Alexander & Senne 2008). The replication of virulent NDV strain in visceral organs causes tissue damage, such as lesions in brain, hemorrhage and necrosis of the intestinal tract, respiratory and caeca tonsils. Haemorrhage can be found in the craw, heart, skin and eyelids as well (Figure 1).

Table 2. The number of birds and pool sample from 10 subdistricts in Subang with rRT-PCR M test results and number of NDV isolates with antibody titre from individual of NDV detected

District	∑ Birds/*Pool*		∑ *pool* matrix with the result (+)			∑ Isolate was obtained	Type of sample
			Duck/Chicken				
	Duck	Chicken	C	O	C&O	Duck/Chicken	
Ciasem	22/4	2 Kp/1	1/1	0/0	0/0	1/0	C
Pusaka Negara	24/5	-	2/0	0/0	0/0	1/0	C
Sukasari	22/5	-	5/0	0/0	0/0	1/0	C
Tambak Dahan	31/7	57 Kp /12	0/6	0/1	0/1	0/2 Kp	2C
Binong	-	15 L /3	0/0	0/0	0/0	0/0	-
	-	86 Br /13	0/3	0/3	0/8	0/4 Br	4O
Compreng	-	20 Kp /4	0/1	0/0	0/1	0/2 Kp	1C & 1O
Pagaden	-	15 Kp /3	0/1	0/0	0/1	0/3 Kp	1C, 1C&1O
Cipunagara	23/4	18 Br/3	0/0	0/0	0/0	0/0	-
	-	153 Br. PS /22	0/4	0/4	0/5	0/2 Br.PS	2O
Cipeundeuy	27/5	-	0/0	0/0	0/0	0/0	-
Subang	-	27 Kp /6	0/3	0/0	0/2	0/2 Kp	2C
Total	149/30	393 / 67	8/19	0/8	0/18	3/15	

Kp= kampong; L= layer; Br= broiler; PS= parent stock; C= cloaca; O= oropharynx

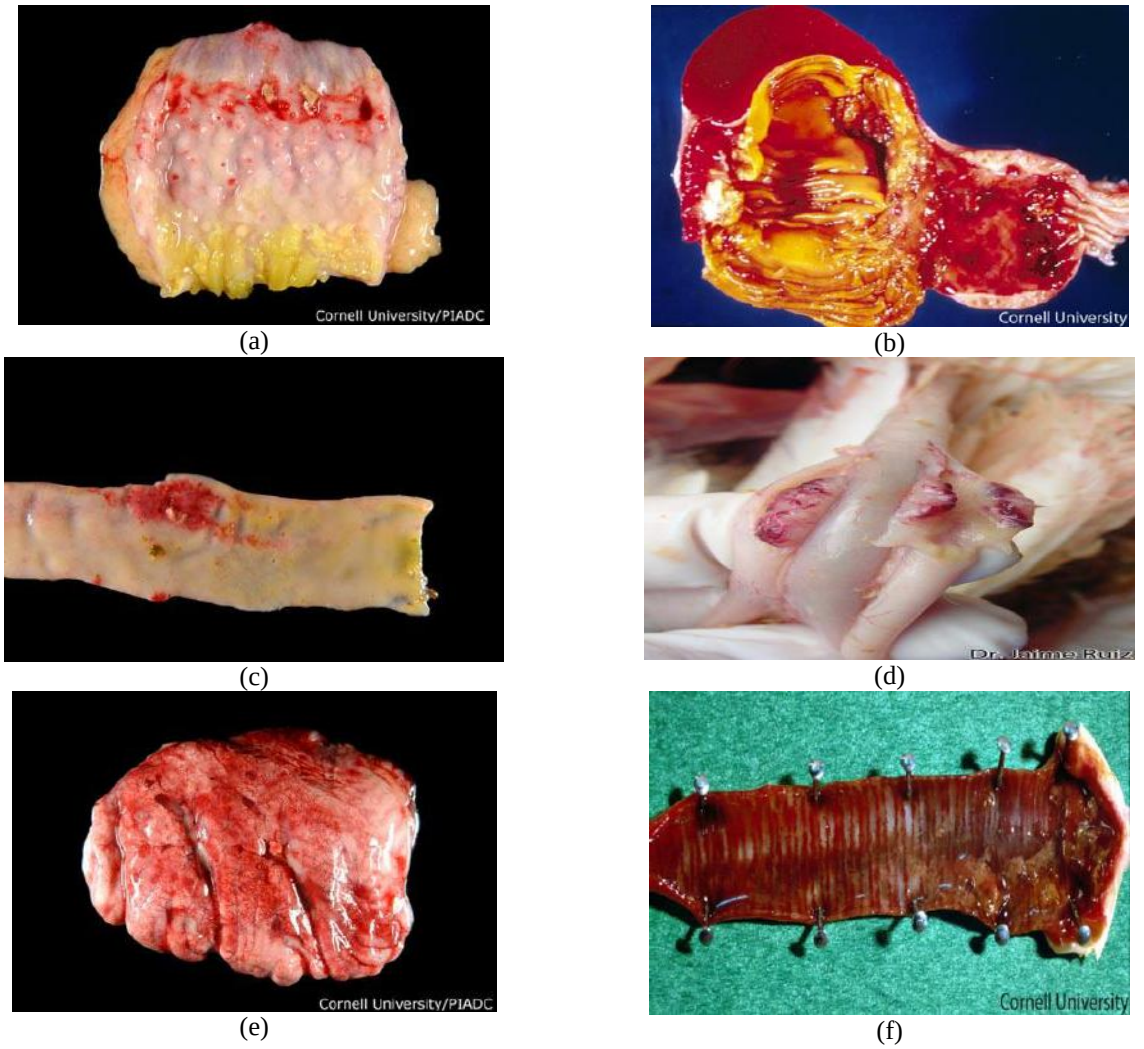
According to Pertulla (2009) percentage of mortality due to infection of velogenyc NDV strain could reached 90% and usually the infected birds will die in 1-2 days after infection. Isolation NDV from swabs of bird cloacal and oropharynx samples from the field in Bangladesh have been done by Haque at al. (2010), and the result showed that 18 isolates were obtained from 20 cloacal swabs and 17 isolates from 20 oropharynx swabs.

ND virus found in unvaccinated native duck was originated from natural infection and usually mesogenyc or velogenyc NDV strain infection in duck showed no clinical symptoms (Saepulloh & Darminto 2005). The NDV isolated from unvaccinated native chickens was also from natural infection. Detection of NDV with RT-PCR has been performed by Kencana et al. (2012) from 10 native chickens in acute-field case with short incubation period (1-2 days) reported and the result showed ND positive. Additionally, Adi et al. (2010) also succed in isolating velogenic NDV strain from native chicken when ND outbreak occur in Bali, and stated that keeping of free-range chicken tends to increase the opportunities for contact with other poultry which has potential risk in the transmission of ND. NDV also found in broilers and parent stocks which had been vaccinated. The chickens were still infected by ND even though it had been vaccinated. It seemed that the vaccination had been carried out was less effective. According to Dortmans et al. (2012), the main cause of the failure of vaccination is caused by improper vaccination implementation and also vaccinated chickens with low antibody usually vulnerable to NDV infection. While according to Hu et al. (2011), the NDV still found in vaccinated chickens because of the shedding of vaccine virus which not homologous with field virus. This was evidenced by Miller et al. (2013) experimentally by vaccinating chickens with LaSota vaccine, and then the chickens were challenged with heterologous virulent NDV (CA/2002) which different genotype with the vaccine virus and the result showed the challenge virus was still excreted into environment and infect unvaccinated chickens (control). According to Miller et al. (2013) using homologous vaccine with field virus may decrease excretion of virus into

environment more than heterologous vaccine. Most of ND vaccine not prevent vaccinated birds from virulent NDV infection but vaccination significantly may decrease the amount of virus excreted through saliva and feces compared to unvaccinated birds (Kapczynski & King 2005; Miller et al. 2009).

NDV was successfully isolated from ducks and chickens showed with and without illness symptoms. According to Emilia (2013), virus was found in the sample from the birds that did not show clinical symptom, possibly due to effect of partial infection in birds, so clinical symptom did not appear, however the virus still excreted. Saepulloh & Darminto (2005) stated that if the NDV can be detected in sick bird feces (cloaca), then this indicated of systemic infection.

Only 18 isolates successfully isolated from 204 (9%) swabs of cloacal and oropharinx that positive of M and inoculated into embryonated chicken eggs (Table 1), it was due to a lot of NDV did not multiplicate in eggs because of the virus already inactive due to the handling and transport of samples were unfavorable. A similar incident also occured in Emilia (2013) study, from 20 samples of individual that positive of gen matrix (M) which were inoculated in eggs, only 11 isolates were successfully isolated. This showed that the tRT-PCR test may detect inactive virus, according to Indriani et al. (2014) one of the advantages of rRT-PCR is able to detect genetic materials of virus either active or inactive. Detection of Antibody with Hemagglutination Inhibition Test (HI).



(a) (b) (c) (d) (e) (f)

Figure 1. Patognomonis pathological alteration of Newcastle Disease infection. *Ptechie* in proventriculus (a); ventriculus (b); intestine (c); caeca tonsil (d); oropharhynx (e); and lung (f) (Buckles et al. 2005)

HI test is often used in laboratories to examine specific antibody titres against NDV because its more specific and does not require special equipment so more economic (Syukron et al. 2013). The serums tested were selected based on representation of the flocks which detected positive and negative of NDV with rRT-PCR from each area. Distribution of antibody classified into three, namely: group 0, meaning that antibodies were not detected, <3, meaning low and no protective antibodies, and ≥3 groups, meaning that protective antibodies (Boven et al. 2008; Rezaeianzadeh et al. 2011).

HI test result on 403 serums from 10 areas showed that 48 serums (12%) containing varied titre antibody; 37 with ≥3 Log2 titre and 11 with <3 Log2 titre (Table 3). Antibodies against NDV detected in waterfowl (ducks), in the chickens were not vaccinated, and in vaccinated domestic birds. Antibodies against NDV detected in 9 of 18 duck serums from Pusaka Nagara area tested the spreading of 8 serums with ≥3 log2 titre and 1 serum with <3 Log2 titre, at 1 of 17 ducks serum and 1 of 32 chicken serums tested from Tambak dahan area with each ≥3 Log2 titre, at 10 of 62 broiler serums from Binong area tested with the spread of 5 serums with ≥3 log 2 titre and 5 serums with <3 log2 titre. in 1 of 13 chicken serum from Pagaden area tested with ≥3 log2 titre, in 26 of 135 broiler breeders (parents stock) serum from Cipunagara area tested with the spreading of 21 with ≥3 log2 titre and 5 with <3 log2 titres. Sample of cloacal and oropharyngeal swab and serum taken from a total of 27 ducks from Cipeundeuy area not found for NDV and content of specific antibodies against ND. This shows that duck samples from Cipeundeuy area were free from NDV. However, to ascertain whether the Cipeundeuy area is free of ND, it is necessary to do the detection and isolation of NDV and detection of specific antibodies against NDV in ducks and other birds such as native chicken and purebred chicken from other locations in Cipeundeuy area.

The percentage of total chicken serum that positively detected specific antibody against NDV (17%) was higher than the waterfowl (10%) and native chicken (2%). In vaccinated broilers, out of 212 serums tested only 36 serums containing antibodies against NDV, which 10 of them (28%) showed a low titre. This is because the serums were taken from culled broiler breeders which was not re-vaccinated of ND (live and killed vaccine) so antibody titre had been decreased, besides the serums were taken from broilers which had just only once ND vaccination and performed at day old with spray method using live ND vaccine and was not re-vaccinated (booster), so the possibility of uneveness of the titres is caused of antibody began to decline.

In waterfowl, only 3 isolates were successfully obtained from 8% positive of M cloacal pools and no isolates obtained from 1% positive of M oropharynx pools. In native chicken, it were 7 isolates obtained from 17% positive of M cloacal pools and 2 isolates obtained from 16 positive of M oropharinx pools. In broiler, there was not isolate obtained from 20% positive of M cloacal pools and 6 isolates obtained from 19% positive of M oropharinx pools (Table 3). There were not a lot of isolates of NDV were obtained, even there was some that not successfully obtained from positive of rRT-PCR M cloacal and oropharinx pools from flocks were also positively detected antibodies against NDV.

Value of coefficient of variation (CV) may used to describe the distribution of antibody titres in groups of animals. Mean and distribution of antibody titres in broilers using Geometric Mean Titre (GMT) and Coefficient of Varian (CV) calculations may be seen in Table 3.

Examination of 212 serum samples of native chicken from Binong and Cipunagara area showed that the average titres were low, ranging from 0-2.8. CV value of the lowest antibody titres was seen in flock of broiler chickens aged 3 weeks from the Binong area. It was 45.3% and the highest seen in culled PS broiler chickens flocks in Cipunagara. It was 185%, while the layer chicken flocks and broiler flocks from the Binong area and in broiler flock in Cipunagara area showed the mean titre of 0 and titres distribution of 0, due to the antibodies in the serum was not detected. CV value of antibody titres from broiler flock in Binong area (45.3%) and broiler flock in Cipunagara area (185% and 49.9%) were look ≥35%. This shows that distribution of antibody titres uneven well. The results of the average titre and distribution of antibody titres of vaccinated broilers in Binong and Cipunagara area were low and did not spreading well. It showed that vaccination of ND in broilers in both of Binong and Cipunagara subdistricts were not optimal.

HI titre showed the immunity status of bird. In unvaccinated native bird and did not show illness symptom, the existence of antibody indicated that the bird had been ever exposed by NDV (Alexander et al. 2004). In vaccinated birds with antibody titre at ≥3 Log2 level indicated a protective antibody. Herd immunity in a population is very important to be protected from the NDV. According to Boven et al. (2008) herd immunity will be obtained if 85% or more of antibody titre was at ≥3 Log2 level after twice vaccination. Kapczynski & King (2005) reported a field case which showed that only birds with anti body titre at ≥4 Log2 level and flock which has group immunity at 66% minimal which resistant to virulent NDV infection after many times vaccination. Generally, antibody titre ≥5 Log2 was considered the most protective. A phenomenon where flock of birds with high antibody titre is still may infected by virulent NDV or opposite of

Table 3. The result of detection antibody specific against NDV in bird serums from 10 areas in Subang area according to poultry commodities

Type/commodites	% Pool M +		Isolate		Programe Vaccination	∑ Serum	∑ +	% +	Antibody titre		
										Log2	
	C	O	C	O		Tested			0	<3	≥ 3
Waterfowl	8	1	3	-	No	102	10	10	92	1	9
Native chicken*	17	6	7	2	No	94	2	2	92	-	2
Purebred chicken	20	19	-	6	Yes	212	36	17	176	10	26
Total	45	26	10			408	48	12	360	11	37

C= cloacal; O= oropharynx; *the native chicken in Subang area were not vaccinated of ND since 2004

Table 4. The result of mean and distribution antibody titre of vaccinated broiler in Subang area using geometric mean tire (GMT) and coefficient of variation (CV)

Subdistrict	Type of chicken	Age	∑ Population (individual)	% *Pool*+ M		∑ Tested serum	Range Antibody titre	GMT	CV
				C	O		(Log2)	(Log2)	%
Binong	Layer	40 mg	120	0	0	15	0	0	0
Binong	Broiler	2 mg	10.000	80	20	35	0	0	0
Binong	Broiler	3 mg	20.000	75	87	50	0–7	0.8	45.3
Cipunagara	Broiler	2mg	18.000	0	0	8	0	0	0
Cipunagara	Broiler PS	culled	14.150	64	27	30	0–5	1.1	185
Cipunagara	Broiler PS	culled	950	40	70	64	0–8	2.8	49.9
Total						212			

CV ≤35% = sebaran of homogenous titre; CV >35% = sebaran of not homogenous titre

it, where the flock with low antibody titre is protective against NDV, in field was still occur often (Yan et al. 2011). Besides, according to Reynolds & Maraga (2000) and Erf (2004) experimentally showed there was no directly correlation between antibody titre in serums against chicken immunity which was challenged with NDV.

Antibody titre response is strongly influenced by quality of vaccine, route and the implementation of application, the environment, individual's factor and the species of bird (OIE 2012). Massal vaccination using live vaccine is often used than individually vaccine, because it is cheaper and easier to be applied (Senne et al. 2004). Vaccine from virulent strain (LaSota and B1) is commonly used around the world to overcome ND because it can provide protection to virulent NDV if the vaccination is done correctly (Kapczynski & King 2005; Cornax et al. 2012; Dortmans et al. 2012). In reality, sometimes the results are not as expected, mass vaccination using spray method showed that the percentage of group immunity was 53%, whereas vaccination by drinking water showed that the percentage of group immunity was 60% (Degefa et al. 2004). Ineffective vaccination also may be affected by immunosuppressive conditions of birds (Perozo et al. 2012). Beside live vaccine, inactive vaccine is also often used in layer and breeder farming because it may provide longer high titre than the live vaccine and the antibody can be downgraded to their generation (Al-Garib et al. 2003). But inactive vaccine is expensive and its application must be done individually, so not all breeders can use it. Alike serology survey in vaccinated broiler and layer chicken in Faisalabad was also done by Numan et al. (2005), the result showed that antibody titre was varies in two types of the bird, but the majority antibody titre was ≥3 Log2. Aziz & Ahmed (2010) did

the serology survey of unvaccinated domestic chicken in Sulaiman Province, Irak. The result showed that serum of 500 tested chickens, 172 serums (34%) was detected antibody. This shows that ND still endemic in Irak.

The existence of a positive antibody specific to NDV in unvaccinated duck and native chicken serums showed that in Subang was vulnerable of ND. This is because the duck and chicken were positive for antibody against NDV may be a carrier which became a source of Newcastle disease spreading (Saepulloh & Darminto 2005; Adi et al. 2010).

Observing the presence of antibodies in 16 birds that excreting NDV from 9 sub districts showed that only 4 individual from 3 sub districts were positive with varying titres (Table 5). The antibody was detected in unvaccinated duck and did not show symptoms of illness from Sukasari area with antibody titre 2 Log2 (isolate 3), two weeks old vaccinated broiler chicken with illness symptom from Binong area with titre 3 Log2 (isolate 7), three weeks old vaccinated broiler chicken with illness symptom with titre 8 Log2 (isolate 8) and 12 weeks old vaccinated breeding broiler (parent stock) and showed illness symptom with titre 5 Log2 from Cipunagara (isolate 13) (Table 5). The existence of antibody in unvaccinated duck and was successfully isolated of NDV showed an immune response of ongoing NDV infection, while the detection of antibody in unvaccinated chickens and ducks where NDV was not found, according Rezaeianzadeh et al. (2011), it showed that NDV infection that has passed and the birds were able to survive and recovery from NDV infection. In the unvaccinated birds where NDV was not detected, according to Daulay (2005) who was successfully isolated NDV from 2 wild birds (pigeon and turtle doves) showed antibody titre 0 Log2 was due to infection process has not been occurred, so the antibody has not been produced. In 3 vaccinated domestic chickens and detected of antibody against NDV, showed high titre antibody in the range 3-8 Log2 may still excrete virus through the cloaca and oropharynx. This may be affected by ineffective vaccination due to vaccine virus with field NDV was not homologous, so virus shedding occurred. Antibody response was not formed due to suboptimal vaccination, so that the chickens may be infected by NDV according to no detected antibody of vaccinated domestic chickens and isolated of NDV. The result of testing sample serum by HI test which successfully isolated from NDV may be seen in Table 4.

Antigenic diversity

Homologous antiserum will has an higher affinity with the viral surface epitopes so its more optimal inhibiting hemagglutination activity. Characterization of 18 antigen isolates with the HI test using antisera Lasota (lentogenic) obtained the average (mean) of HI titres between 6-8 log2 with antisera and Komarov (mesogenic) between 9-13 log2. Isolates affinity with LaSota antisera showed relatively homogeneous antigenic character, only a few isolates that showed variation reaches 2 log2 (1st, 2nd, and 3rd isolate), as well as antisera Komarov, only a few isolates that showed variation reaches 4 log2 (3rd, 6th, and 16th isolate). All NDV isolates showed a higher affinity against antisera Komarov compared with Lasota antisera that indicates all the NDV tend to get into a virulent NDV strain (Figure 2). According to Alexander & Senne (2008) differences of antigenic between NDV strains which may be recognized by specific antibodies determined by a hemagglutinin (HN) protein. Beside it, according Adu (1985) and Ibu et al. (2008) antigenic variation in NDV from the same strainoccurs because of various functions of the external proteins due to mutation.

Study of the antigenic diversity of NDV using polyclonal antisera had been conducted by Emilia (2013) using antisera strain Lasota, Komarov, the G7 and the ITA against four isolates Serpong area, West Java. The result showed that varies affinity with HI titres ranging between 3-5 log2 (Lasota), 5-10 log2 (Komarov), 6-8 log2 (G7) and 3-7 log2 (ITA). Hsiang-Jung et al. (2004) examined the variation of antigenic against 36 isolates from Taiwan NDV obtained between 1969-1996 using 22 monoclonal antibodies (MAB) and was able to separate the 36 isolates into 18 groups antigenic and based on the nucleotide sequences of gene F were grouped into 15 genotypes. Characterization of NDV antigen may also be done with a monoclonal antibody (MBA). Hu et al. (2010) showed using four types of MBA, that NDV antigenic variation may occur due to mutations of residues K (Lysine) at position 347 in the HN protein.

Characterization of physical properties of ND Virus with Elution Time Test

Determination of NDV strain may be known by its biological activity, including by elution time test. Significantly different in the time value may be used to distinguish of NDV strain on field roughly. According to Ezeibe & Ndip (2005) elution time of velogenic patotype virus had elution time between 84-189 minute, while mesogenic virus had elution time between 45-78 minute and virus that includes lentogenic (LaSota) had elution time for 20-43 minutes. Characterization of the pathogenicity of 18 isolates with elution showed 3 isolated include mesogenic group and 15 isolates to velogenic group (Table 5). Observing the elution time of isolates obtained from cloacal and oropharyngeal swabs from 1 native chicken in Compreng area (isolate

Table 5. Result of detection of specific antibody against NDV with HI test in 16 bird's serums which successfully isolated of NDV in Subang area

District	Type of bird	%	Antibody	Isolate	
		Pool+ M	Titre(Log2)	Code	Type of sample
Ciasem	Duck	40%	0	1	C
Pusaka Negara	Duck	40%	0	2	C
Sukasari	Duck	80%	2	3	C
Tambak Dahan	Kp. chicken	42%	0	4	C
Tambak Dahan	Kp. chicken	42%	0	5	C
Binong	Broiler	69%	0	6	O
Binong	Broiler	69%	3	7	O
Binong	Broiler	69%	8	8	O
Binong	Broiler	69%	0	9	O
Compreng	Kp. chicken	50%	0	10a	C
Compreng	Kp. chicken.	25%	0	10b	O
Pagaden	Kp. chicken	67%	0	11	C
Pagaden	Kp. chicken	66%	0	12a	C
Pagaden	Kp. chicken	67%	0	12b	O
Cipunagara	Broiler Ps.	31%	5	13	O
Cipunagara	Broiler Ps.	31%	0	14	O
Subang	Kp. chicken	83%	0	15	C
Subang	Kp. chicken	83%	0	16	C

Kp= kampong; Ps= parent stock; C= cloaca; O= oropharynx

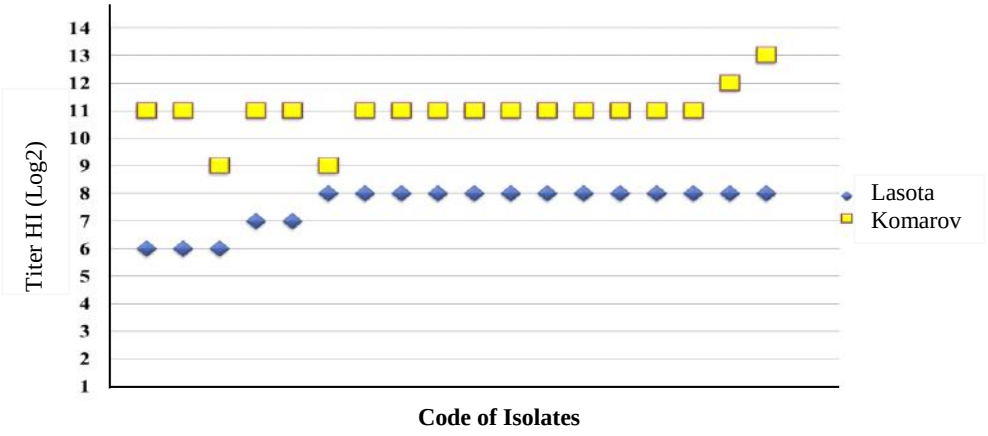


Figure 2. Comparison of antigenic of 18 isolates with HI test. The majority of HI titres looks homogeneous, only a few isolates that showed a variation with the antisera Lasota 2 log2 and with antisera Komarov 4 log2. HI titre comparison of both antisera showed isolates tend to lead to a virulent strain

Table 6. The result of elusion time test of NDV isolates that obtained from chicken and duck in 10 areas in Subang area

District	Type of Bird	Code of isolate	Type of samples	Elusion time (minute)	Type of Patotipe
Ciasem	Duck	1	C	117±0.0	Velogenic
Pusaka Negara	Duck	2	C	119±0.0	Velogenic
Sukasari	Duck	3	C	162±0.0	Velogenic
Tambak Dahan	Kp. Chicken	4	C	165±5.2	Velogenic
Tambak Dahan	Kp. Chicken	5	C	117±0.0	Velogenic
Binong	Broiler	6	O	89.6±23.7	Velogenic
Binong	Broiler	7	O	225±0.0	Velogenic
Binong	Broiler	8	O	232±12.1	Velogenic
Binong	Broiler	9	O	105±0.0	Velogenic
Compreng	Kp. Chicken	10a	C	219±0.0	Velogenic
Compreng	Kp. Chicken	10b	O	262.3±0.0	Velogenic
Pagaden	Kp. Chicken	11	C	117±0.0	Velogenic
Pagaden	Kp. Chicken	12a	C	80±0.0	Mesogenic
Pagaden	Kp. Chicken	12b	O	70±0.0	Mesogenic
Cipunagara	Kp. Chicken	13	O	92.6±21.5	Velogenic
Cipunagara	Kp. Chicken	14	O	76.3±1.2	Mesogenic
Subang	Kp. Chicken	15	C	117±0.0	Velogenic
Subang	Kp. Chicken	16	C	162±0.0	Velogenic

Kp= kampong, 10a and 10b; 12a and 12b= from the same individual

10a and 10b) and isolates obtained from cloacal and oropharyng swabs from 1 native chicken in Pagaden area (isolate 12a and 12b) were included to the same strain that was mesogenic/mesogenic and velogenic/velogenic, and HI titre from each isolate also had the same value. It indicated that both of excreted isolates from cloacal and oropharinx from 1 individual were the same NDV, but to confirm the virus identity, it needs to be sequenced. Elution time may be affected by temperature. According to Hussain et al. (2008) the elution time will be longer at 4°C and will be faster when the temperature is raised, therefore in order to obtain an accurate results, the elution test should be performed at a steady temperature. Besides, the elution test was also influenced by the concentration of red blood cells, if there are too many red blood cells during the testing, the red blood cells are not able to be bound by the virus, so that looks like a reaction to release of red blood cells by the virus and this will reduce the efficiency of elution test (Ezeibe & Ndip 2004).

Characterization of ND Virus by rRT-PCR fusion (F)

Characterization of 18 isolates by rRT-PCR fusion (F) showed positive result for all isolates, indicating that all isolates were virulent virus strain. The result of rRT-PCR F test showed there was a correlation with the result of elusion time test where was obtained mesogenic isolates and 15 velogenic isolates, this is correspond with the result of rRT-PCR F that showed all isolates were positive for virulent NDV strain. Not all virulent NDV may be detected by rRT-PCR using primer fusion (F). According to Kim et al. (2006) specificity in molecular diagnostic tests, may be affected by nonconformities between nucleotide pairs of primer base and probe with sequence that have a potential to give a fake negative result, while according to Cattoli et al. (2011) high degree of nucleotide variation in gen F may cause incompatibility between the primers and probes with the NDV amino acid

sequence. The incompatibility of primer oligobucleotide cause hybridization between primer/probe with RNA of virus not occurs, so it was not detected by real-time PCR system software.

CONCLUSION

Newcastle disease was still endemic in Subang area and the infection may be subclinical. Eighteen ND viruses which were found, majority had relatively homogeneous character, just a few isolates that showed diversity of pathogenicity and antigenicity (antisera LaSota: isolate 1, 2, and 3; antisera Komarov: isolate 3, 6 and 16). The ducks from Cipeundeuy area had not been exposed of NDV. The distribution of antibody titres in vaccinated chicken was unequal. The result of this study contributed information about ND in Subang area and may be used as feedback for Subang government to determine the prevention and control programs of ND infection in poultry in Subang.

RECOMMENDATION

Further research needs to be done to see the acid-base sequences of cleavage site of fusion (f) protein by sequencing or *pathotyping* test (MDT, ICPI, and IVPI) in order to obtain more information about pathogenicity and NDV strains which spread in Subang area. Sequencing needs to be done to see mutation in hemaglutinin. Strict sanitary and vaccinate use a combination of homologous live and inactive vaccine with field NDV can be applied to prevent ND infection in birds in Subang. Besides, more extensive surveys with more samples needs to be done to get information about infection of NDV that cover all areas in Subang area.

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Curcumin Effect on Bleomycin-Induced Pulmonary Fibrosis in *Mus musculus*

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ABSTRAK

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Kurkumin merupakan bahan aktif utama dari tanaman kunyit (*Curcuma longa*) diketahui memiliki aktivitas sebagai anti-oksidan dan anti-inflamasi. Bleomisin merupakan salah satu obat anti-kanker yang dapat menginduksi fibrosis paru-paru pada manusia dan hewan. Tujuan penelitian ini adalah untuk mengetahui efek biologis kurkumin pada fibrosis paru-paru yang diinduksi bleomisin pada mencit. Sebanyak 16 ekor mencit galur ddy dibagi dalam 4 kelompok perlakuan: (i) kontrol, 100 µl aquadest steril diinjeksikan secara SC, (ii) bleomisin (BLM), 100 µl bleomisin konsentrasi 1 mg/ml diinjeksikan secara SC, (iii) kurkumin (CMN), 100 µl aquadest steril diinjeksikan secara SC dan 100 mg/kg BB kurkumin dalam 0,5% carboxymethylcellulose (CMC) yang diinjeksikan secara IP, dan (iv) BLM+CMN, 100 µl bleomisin dengan konsentrasi 1 mg/ml diinjeksikan secara SC dan 100 mg/kg BB kurkumin dalam 0,5% CMC diinjeksikan secara IP. Semua perlakuan diberikan setiap hari selama 4 minggu. Organ paru-paru dikoleksi dalam 10% buffered neutral formalin (BNF). Pengamatan histopatogi dengan pewarnaan hematoksilin-eosin (HE) dan Masson's trichrome (MT) untuk diamati tebal dinding alveol dan luas daerah jaringan ikat. Hasil penelitian menunjukkan bahwa pada kelompok perlakuan bleomisin (BLM) terjadi peningkatan luas jaringan ikat dan tebal dinding alveol secara signifikan jika dibandingkan dengan kontrol. Sementara itu, pemberian kurkumin pada mencit yang mendapatkan induksi bleomisin (kelompok BLM+CMN), menyebabkan terjadinya penurunan signifikan luas jaringan ikat dan tebal dinding alveol. Dapat disimpulkan kurkumin memiliki aktivitas sebagai inhibitor fibrogenesis untuk mengurangi keparahan fibrosis paru-paru akibat aplikasi bleomisin pada mencit.

Kata Kunci: Bleomisin, Fibrosis Paru-paru, Mencit, Kurkumin

ABSTRACT

Rahmi A, Setiyono A, Juniantito V. 2015. Curcumin effect on Bleomycin-induced pulmonary fibrosis in *Mus musculus*. JITV 20(2): 148-157. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1169

Curcumin, a curcuminoid compound of turmeric has been demonstrated to have anti-oxidant and anti-inflammatory properties. Bleomycin (BLM) is an anti-cancer drug induced pulmonary fibrosis in human and animals. This study was aimed to investigate biological effects of curcumin on bleomycin-induced pulmonary fibrosis in mice (*Mus musculus*) through pathomorphological assessment. In this study, 16 mice ddy strain were divided into four groups, namely (i) control, mice were subcutaneously (SC) injected with 100 µl sterilized aquadest in dorsal skin, (ii) BLM group, injected SC with 100 µl of 1 mg/ml BLM in dorsal skin, (iii) Curcumin (CMN) group, mice were intraperitoneally (IP) injected with 100 mg/kg body weight (BW) curcumin dissolved in 0,5% carboxy methyl cellulose (CMC) and injected with 100 µl sterilized aquadest SC, (iv) BLM+CMN group, injected SC with 100 µl of BLM 1 mg/ml and injected IP with 100 mg/kg BW CMN in 0,5% CMC. All treatments were performed daily for four weeks period. The lung samples were collected and fixed in buffered neutral formalin (BNF) 10%. Histopathological evaluation was performed with hematoxylin-eosin (HE) and Masson's trichrome (MT) stains. The results showed that BLM treatment significantly increased fibrosis area and alveolar wall area fraction as compared to control. OIn the other hand, CMN treatment significantly reduced fibrosis area and alveolar wall area fraction in mice treated with BLM. In conclusion, our study showed that CMN treatment may inhibit lung fibrogenesis in BLM-induced pulmonary fibrosis.

Key Words: Bleomycin, Curcumin, Mice, Pulmonary Fibrosis

INTRODUCTION

Pulmonary fibrosis is a chronic pulmonary disease characterized by pathological lesions in the form of extracellular matrix and tissues component changes, followed by clinical symptoms, physiological disorders, and radiographic findings (Todd et al. 2012). Many factors cause this pulmonary fibrosis, such as it is exposured by air poison, particular pulmonary disease, effect of radiation therapy and chemotherapy (Ley & Collard 2013).

Bleomycin (BLM) is used as a chemotherapy agent for various cancers. This antibiotic of antitumor is a derivative glycopeptide isolated from *Streptomyces verticillus* (Yamamoto 2010). BLM has strong antitumor activity. Clinically, BLM is used as tumor therapy such as *squamous cell carcinoma* at around head and neck (including mouth, tongue, tonsils, nasopharynx, oropharynx, sinus, palate, lips, buccal mucosa, the gums, the epiglottis, and larynx), *malignant lymphoma, testicular carcinoma,* and *malignant pleural effusion* (Chu et al. 2010).

The main limitation of BLM therapy is its side effect which causes alveolitis fibrosis (Matsushita et al. 2008). Frusch et al. (2012) said that there are several syndromes in the lung linked with BLM utilization, that is bronchiolitis obliterans, hypersensitivity eosinophils, and interstitial pneumonitis which will thrive become pulmonary fibrosis. Besides, BLM-induced pneumonitis can reach 46% in patients who receive BLM treatment. Mortality level of the pulmonary fibrosis disease is around 10-20% with 2-3% from patients treated by BLM. Brugge et al. (2013) said that BLM therapy side effect could cause pneumonitis.

Fibrosis mechanism in lung due to the side effect of BLM utilization has not known. Some factors that have role in pulmonary fibrosis in BLM therapy are oxidative stress, BLM-hydrolase enzyme deactivation, genetic, and the release of inflammatory cytokines (Brugge et al. 2013; Reinert et al. 2013). BLM use in human also causes other side effect, such as an abnormality skin characterizes the scleroderma (Juniantito et al. 2013).

Fibrosis pathogenesis (fibrogenesis) is divided in several overlapping phases, namely inflammation, connective tissue proliferation, and intractable fibrosis (Matsushita et al. 2008; Reinert et al. 2013). Retardation in one step of fibrogenesis causes a decreasing of connective tissue formation (Loomis-King et al. 2013). BLM could stimulate endothelial cells, macrophages, and fibroblasts to induce synthesis of inflammatory mediators especially proinflammatory and fibrogenic cytokines, inducing apoptosis, and free radicals synthesis (Yamamoto 2010; Yamamoto & Katayana 2011). It was thought that prevention of fibrosis might be mediated by inhibition of inflammation with anti-inflammatory substances (Basnet & Skalko-Basnet 2011; Kardena & Winaya 2011).

Curcumin (diferuloylmethane) is an active ingredient in turmeric, in addition, it is also known as an antioxidant (Zhang et al. 2011). Turmeric is widely available plant in Indonesia which is used often as spice and herbal ingredient. Anti inflammation effect of curcumin is likely because of a high pleiotropic molecule which able to interact with and related to the most transcription factors in inflammatory mediators synthesis and inhibiting free radicals releasing in the inflammatory cells (Jurenka 2009; Marçal et al. 2012).

Some studies have been conducted to determine curcumin effect as anti-inflammation and its potential to prevent the fibrosis (Jurenka 2009; Beevers & Huang 2011; Kardena & Winaya 2011). Curcumin can decrease fibrosis level in liver, kidney, and lung of laboratory animal (Beevers & Huang 2011). Therefore, curcumin can be used as potential candidate of anti-fibrosis preparate, especially in pulmonary fibrosis. This study was aimed to see the curcumin potential in preventing the side effect of BLM use in cancer patients who are undergoing a chemotherapy.

MATERIALS AND METHODS

Time and place

This study was conducted during December 2014 - March 2015 in Laboratory Animals Management Unit (UPHL), Faculty of Veterinary Medicine, Bogor Agricultural University and Histopathology Laboratory, Pathology Division, Faculty of Veterinary Medicine, Bogor Agricultural University.

Inducer material of pulmonary fibrosis

Bleocin® (Bleomycin hydrochloride 15 mg, Kalbe Farma, Jakarta, Indonesia) was diluted in 15 ml sterile aquadest to reach concentration of 1 mg/ml. As much as 100 µL of the solution was injected subcutaneously (SC) on the back skin for BLM and BLM+CMN group everyday for 4 weeks.

Curcumin

Curcumin active ingredients (Biopurify, Chengdu, China) was diluted in 0.5% *carboxymethylcellulose* (CMC) and injected 100 mg/kg of body weight by intraperitoneal injection (IP) in CMN and BLM+CMN group everyday for 4 weeks.

Experimental procedure

All of procedures conducted in this study met the requirement of Animal Ethics Commission of Bogor Agricultural University Number 25-2014 IPB. Sixteen 4 weeks old male ddy strain mice with body weight around 20-25 gram (The National Agency of Food and Drug Control (NA-DFC), Jakarta, Indonesia) were used and divided into 4 groups. There were 4 mice in each group.

This study was divided into 4 treatment groups, namely: (i) control, injected subcutaneously by 100 µL sterile aquadest in the back skin, (ii) BLM, injected

subcutaneously in the back skin with 100 µL BLM of 1 mg/mL concentration, (iii) CMN, injected subcutaneously with 100 µL sterile aquadest in the back skin and injected intraperitoneally with curcumin of 100 mg/kg of body weight in 0.5% CMC, and (iv) BLM+CMN, injected subcutaneously with 100 µL BLM of1 mg/mL concentration and injected intraperitoneally with curcumin of 100 mg/kg of body weight in 0.5% CMC. Injection of the curcumin by intraperitoneal injection was adapted from Li et al. (2013).

This study was done in three steps, namely: (i) acclimatization, the experimental animals were adapted in new cage for 2 weeks, (ii) treatments, the experimental animals were treated in accordance to the respective groups everyday for 4 weeks, (iii) termination, the experimental animal were euthanized by giving Ketamine HCL of 0.2 mL/head (AVMA 2013). Furthermore, left lobus of lung was collected to be made histopathology preparates, then stained by hematoxylin-eosin (HE) for observation of tissue structure of the lung (Fischer et al. 2006) and Masson's*trichrome* (MT) to see the presence of connective tissue (Suvik & Effendy 2012).

Histopathological assesment by HE and MT stains

Lung of BNF 10% was sliced in 3 mm and inserted into *tissue cassette* for dehydration process, clearing and paraffin infiltration using automatic tissue processor. Chunk organs, further was printed in the paraffin until paraffin block formed. For histology test, the paraffin block was sliced by rotary microtome in 3-5 µm of thickness. Cutting results were placed in object glass to be deparafinized and rehydrated for tissue staining by hematoxylin-eosin and Masson's *trichrome*. All of the cutting results were observed by a light microscope which was connected to the computer. Observed parameters of this organ were connective tissue width and alveoli wall thickness.

Connective tissue width

Connective tissue width was quantitatively counted by *Image J®* software (http://imagej.nih.gov//ij/; NIH, Maryland, USA), by analyzing tissue slide which has stained by modification of MT stains (Suvik & Effendy 2012). *Image J®* software utilization is to decrease the level of counting subjectivity by scoring method. By using 40x objective lens magnification, as much as 20 visual fields per treatment group randomly selected by video camera (Indomikro® HDMI camera) which was shown on colored screen. The width of each visual field was 326.40×184.00 µm^2. Furthermore, figure was customized for contrast, brightness, and threshold color. Image analysis program detected the width of blue area which indicates collagen area or connective tissue in each visual field and presented in the form of percentage.

Alveolar wall thickness

Alveoli wall thickness quantitatively was counted by analyzing tissue slide which has been stained by HE. As much as 20 visual fields per treatment group were randomly selected using video camera showed on the colored screen. The wide of each visual field was 326.40×184.00 µm^2. Alveoli wall thick measurement was done 10 times of every visual field, and the data presented in the form of average.

Data analysis

Percentage of width of connective tissue and alveoli wall thickness were analyzed by SAS® 1.9 software for *Microsoft®Windows®* ANOVA and presented in form of average and standard deviation. Further, Duncan advance test was done to determine whether there is a significant differences between the treatments or not.

RESULT AND DISCUSSION

Histology Result by Hematoxylin-Eosin Staining

Figure 1 is photomicrography of lung tissue of each treatment. Figure 1A is a photomicrography of control group of lung tissue. In this figure, normal alveoli wall structure is 3.7±0.6 µm. The same thing occurred in CMN group (Figure 1C). It is shown that lung with normal alveoli wall structure of 3.4±0.5 µm.

Figur 1B is a photomicrography of BLM group. In this group, it is shown an inflammation accompanied by an increase in cell number in interstitium, therefore alveoli wall become thicker (24.9±6.3 µm) than the control group. Besides, there was an infiltration of inflammatory cells dominated by macrophages and lymphocytes. Alveoli wall thickening resulted in width of alveoli area became smaller, therefore the air entered the lung became limited. Rienert et al. (2013) said that thickening of the alveoli wall would cause difficulty in breathing (anoxia). In the BLM+CMN group (Figure 1D), it is shown lesion similar to the BLM group, that is the presence of inflammation, increasing in cell number in interstitial and the thickening of the alveoli wall (9.9±2.6 µm) with lower severity compared to the BLM group.

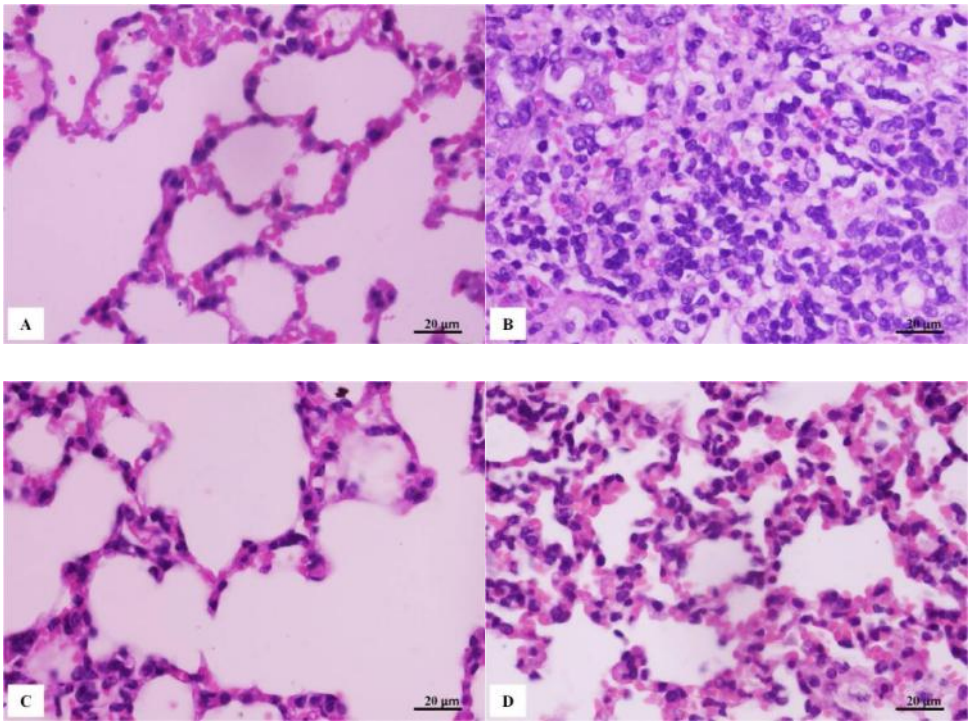

Figure 1. Photomicrography of lung tissues by HE staining of each treatment group: Control (A); BLM (B); CMN (C); and BLM+CMN (D)

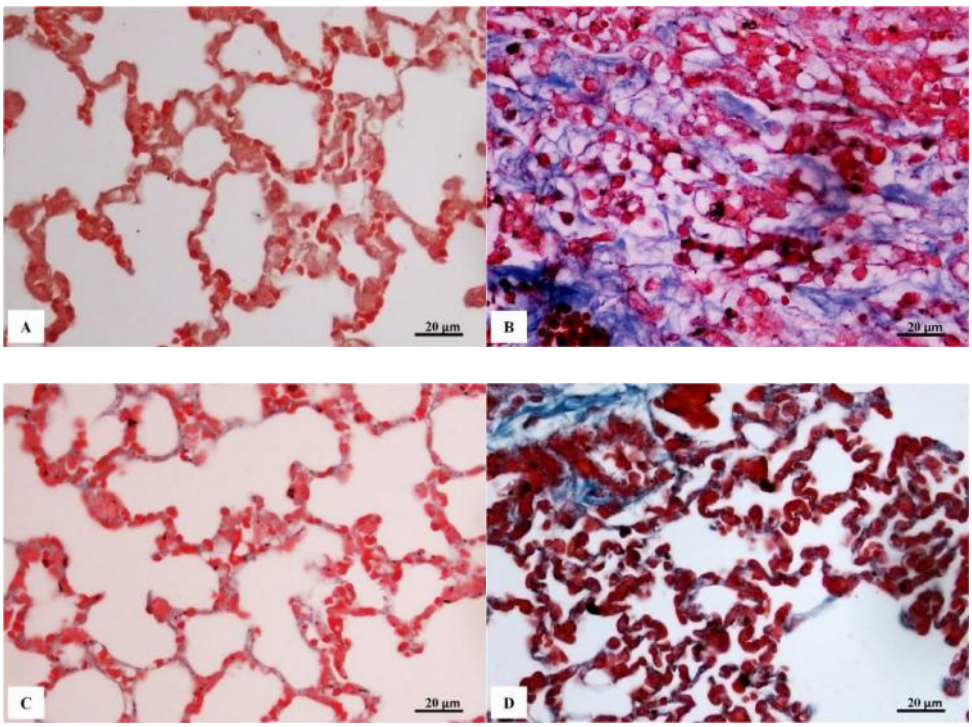

Figure 2. Photomicrography of lung tissues by MT staining of each treatment group: Control (A); BLM (B); CMN (C); BLM+CMN (D)

Yamamoto & Nishioka (2000) said that in mice everyday induced by BLM SC for 4 weeks, there was pulmonary fibrosis characterized by mononuclear cell infiltration, ederma, and septum alveolar thickening due to decrease in number of macrophages alveolar and lymphocyte. Besides, Izbicki et al. (2002) found that there were perivascular and lymphocytes peribronkial (day 3), subcapsular fibrosis (day 6), and decreasing in number of macrophages alveolar and septum alveolar (day 14) in mice's lung histology induced by pulmonary fibrosis with single dose BLM by intratracheal.

Histology result by Masson's Trichrome (MT) staining

Tissue staining techniques by MT is addressed to identify the presence of the connective tissue or collagen in the tissue. Result of the lung tissue staining by MT technique was presented in Figure 2.

Figure 2A (control) and 2C (CMN) shows normal structure of lung tissues with thin alveoli wall (3.7±0.6 μm). In the two figures do not show an excessive blue structure, indicating that in the two group there is no excessive collagen accumulation. On the contrary, figure 2B (BLM) shows blue accumulation which extends almost to the entire visual field. The blue color on lung slide colored by MT indicating the presence of accumulation connective tissue or collagen (Suvik & Effendy 2013). Besides, in the BLM group was also seen smaller alveoli diameter compared to the control group. Figure 2D (BLM+CMN) shows light accumulation of collagen visualized by blue color and looked like thickening of the alveoli wall.

Width of connective tissue

Measurement of connective tissue width in the lung was done to determine extent of lung tissue damage due to BLM application. In figure 3, it is not seen the presence of a significant differences of the connective tissue between control group (2.4±0.8%) and CMN group (1.5±0.6%). However, in BLM group (15.0±2.4%), there was significantly wider connective tissue value ($P<0.05$) compared to control group. In the BLM+CMN group (6.2±2.0%), there was significantly lesser width connective tissue ($P<0.05$) compared to the BLM group. This research result was in line with Izbicki et al. (2002) that there was an increase in connective tissue width of mice lung induced by single dose Bleomycin by intratracheal. Besides, Liu et al. (2013) also reported that there were an inflammation and fibrosis in interstitial of mice lung tissue induced by BLM through intratracheal.

Wide development of connective tissue in BLM group was caused by BLM activity which may induce pulmonary fibrosis by causing inflammation, than it developed become proliferation of fibroblast. Fibroblast is a cell which produced extracellular matrix (Todd et al. 2012). Yamamoto & Katayama (2011) said that BLM can stimulate endothelia cell, macrophage, and fibroblast to induce inflammatory mediator. The inflammatory mediator will induce proinflammatory cytokines, cytokine fibrogenic, transforming growth factor-β (TGF-β), apoptosis, and the release of free radicals.

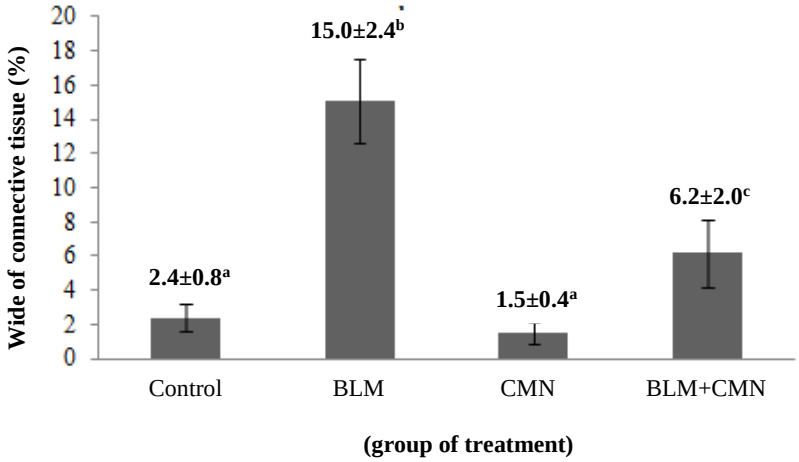

Figure 3. The wide of connective tissue of each treatment group, different superscript word shows significant difference ($P<0.05$)

Reinert et al. (2013) reported that low *BLM-hydrolase* enzyme activity can be a predisposing factor of pulmonary fibrosis due to the BLM application. Through in vivo, the BLM will be metabolized become non-toxic molecule with *Bleomycin-hydrolase* enzyme help (Haston et al. 2002). This enzyme is cytosolic aminopeptidase group which has low activity in lung and skin (Reinert et al. 2013). Therefore, BLM application may cause the pulmonary fibrosis. The fibrosis in the lung is started by an inflammatory. The inflammatory will develop into proliferation of fibroblasts into myofibroblast (cell produces collagen), and it finished by persistent formation of connective tissue. Mechanism of fibrosis induction by BLM was caused by increasing secretion of extracellular matrix proteins because of cytokine fibrogenic effect which was released by macrophages, endothelial cells, and fibroblasts (Reinert et al. 2013). Besides, BLM in the cells will bind with iron ion (Fe^{+2}) and oxygen forming Fe^{+3} ion which produce the free radicals, so that cause DNA damage, RNA degradation, and inducing the apoptosis (Yamamoto 2010). Moreover, the free radicals or reactive oxygen species can produce direct toxicity through participation in redox reactions and subsequent fatty acid oxidation, which leads to membrane instability. Oxidant can cause inflammatory reaction within be lung. The inflammatory mediators like cytokines such as interleukin-1, macrophage inflammatory protein-1, platelet-derived growth factor, and TGF-β are released from alveolar macrophages in animal models of bleomycin toxicity, resulting in fibrosis. Damage and activation of alveolar epithelial cells may result in the release of cytokines and growth factors that slimulate proliferation of myofibroblast and secretion of pathologic extracellular matrix, leading to fibrosis.

The inflammation due to BLM application will cause body to response to repair the tissue by involving connective tissue reconstitution from the fibroblast into myofibroblast (Reinert et al. 2012). Myofibroblast is the main component in fibrosis process or wound healing. This myofibriblast will produce extracellular matrix, so it will cause extracellular matrix proteins accumulation. Previous study conducted by Xu et al. (2009) showed that TGF-β is cytokine inducer of differentiation of fibroblast cell into myofibroblast cell. Yamamoto & Nishioka (2004) presented that several actions of TGF-β are, among other as chemotactic macrophages and fibroblasts, stimulating fibroblast proliferation, increasing extracellular matrix synthesis, decreasing extracellular matrix proteinase regulation, and increasing proteinase inhibitor regulation. So that exacerbates the development of fibrotic lesions in the lung.

The opposite occurred in BLM+CMN group where there was a significant decrease ($P<0.05$) of width of connective tissue compared to the BLM group (Figure 3). In line with Punithavathi et al. (2000) that there was a decreasing of alveolar macrophage amount, superoxide and nitrikoksida in rats given oral curcumin treatment after single dose Bleomycin induction by intratracheal. So, in his research, it was concluded that curcumin was a potential material as an anti-inflammatory and anti-fibrogenic in BLM-induced pulmonary fibrosis.

Bleomycin as an antibiotic antitumor agent, widely used for livestocks, such as pig (Balazs et al. 1994) and sheep (Organ et al. 2015). Same as in laboratory animals, BLM utilization in pig and sheep also caused side effect such as an inflammation into pulmonary fibrosis (Balazs et al. 1994; Organ et al. 2015). Pathogenesis mechanism of pulmonary fibrosis due to BLM application in livestocks was alike with the laboratory animals, so that was possible to be given curcumin as anti-inflammation and antioxidant to reduce fibrogenesis severity in the pulmonary fibrosis cases due to BLM application.

The decreasing of width of lung connective tissue in this study may be caused by 2 factors. First, the curcumin can directly inactivate the bleocymin activity, so that inflammation did not occur. Second, activity of anti-inflammation from the curcumin causes the inflammation become lower, so that fibrosis can be pressed. The second one is closer with pathogenesis of fibrosis inhibition by the curcumin. This was caused by BLM induction and curcumin therapy conducted through 2 different ways. Bleomycin induction was done subcutaneously, whereas curcumin therapy was done by intraperitoneal. Both of subcutaneous and intraperitoneal ways will interact systematically in the body, so that increasing width of lung connective tissue due to Bleomycin application possibly hampered by anti-inflammation activity from the curcumin. Besides, the curcumin was known to have effectiveness as an inhibitor receptor TGF-β, so that TGF-β production can be inhibited (Mo et al. 2012).

Prasad et al. (2014) said intraperitonel injection of curcumin more often applied to animals than to human. Besides, the bioavailability compound of curcumin via intraperitoneal injection is higher than gavage. Interestingly, oral curcumin treatment showed no effect on important measure of BLM-induced injury in mice, whereas intraperitoneal curcumin administration effectively inhibited inflammation and collagen deposition along with a trend toward improved survival of animal, and also reduced fibrotic progression even when administered after the acute bleomycin-induced inflammation had subsided (Smith et al. 2010).

Smith et al (2010) said that curcumin administration could significantly prevent lung inflammation and collagen deposition in pulmonary fibrosis induced by BLM by intratracheal. Curcumin was high pleiotropic

molecule which able to interact and bind with the most of inflammation molecule target (Jurenka 2009; Marçal et al. 2012). Curcumin has been reported having activity as an anti-cancer (Wiken et al. 2011), anti-oxidant (Bhullar et al. 2013) and anti-inflammation (Basnet & Skalko-Basnet 2011). Turmeric bioactive compounds had protective effect to heart cell which exposed to toxic chemicals such as tetrachloride (CCl_4) (Prakash et al. 2008; Kardena & Winaya 2011). Yu et al. (2011) reported that curcumin administration can reduce pancreatic tissue damage and another organs due to *caerulein* induction through inhibiting the release of inflammatory mediator of TNF-α cytokines.

Curcumin has been reported to exhibit anti-tumorigenic and chemo-preventive activities due to the structural resemblance of dibenzoyilmethane (DBM) to the anti-inflammatory and aspirin-like skeleton of DBM derivates (Lin et al. 2011). Villegas et al. (2011) reported curcumin has also demonstrated protective and preventive effect in progression of colorectal colitis cancer, which was collerated with a lowered immune-reactivity of beta-catenin and reduction of pro-inflammatory cytokine levels and a decrease of inflammatory mediator's overexpression.

Lee et al. (2010) evaluated that curcumin as a potential dietary supplement in the setting of thoracic radiotherapy in mice with creating a baseline radioprotective state prior to irradiation by inducing protective gene expression as well as having potent direct antioxidant scavenging activity. Li et al. (2013) said curcumin application has a protective effect on the acute hepatic injury induced by acetaminophen with reduced acetaminophen-induced hepatocyte apoptosis and that protection may be related to its inhibition of lipid peroxidation and oxidative stress.

Thickness of alveolar wall

Figure 4 shows that there is no significant difference of alveolar wall between control group (3.7±0.6 µm) and CMN group (3.4±0.5 µm). However, there was significantly (P<0.05) higher alveolar wall thickness value in BLM group (24.9±6.3 µm) compared to the control group. In BLM+CMN group (9.9±2.6 µm) there was significantly (P<0.05) lower alveolar wall thickness value compared to BLM group.

The results about width of connective tissue and alveolar wall thickness in each treatment group were presented in Table 1.

This research result is aligned with a research of Izbicki et al. (2002) where alveolar wall thickening in mice lung tissue induced by single dose BLM by intratracheal. Alveolar wall thickening in BLM group

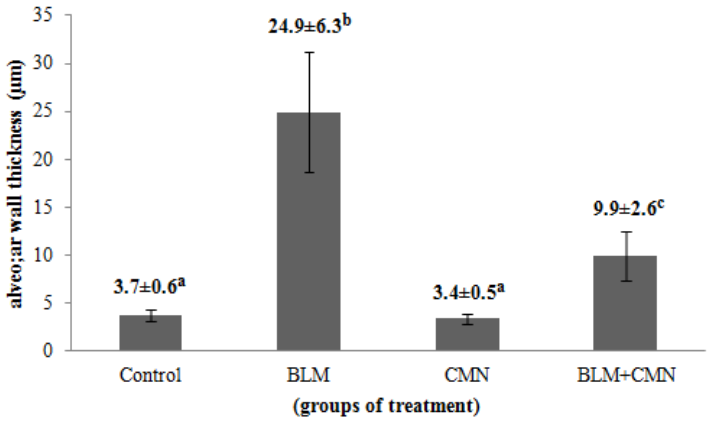

Figure 4. The Alveolar wall thickness in each treatment group, different superscript word shows significantly (P<0.05) difference

Table 1. Result of the wide of connective tissue and alveolar wall thickness

Treatment Group	Wide of connective tissue (%)	Alveolar wall thickness (µm)
Kontrol	2.39±0.78[a]	3.72±0.56[a]
BLM	15.03±2.45[b]	24.93±6.26[b]
CMN	1.50±0.61[a]	3.39±0.54[a]
BLM+CMN	6.17±2.01[c]	9.95±2.60[c]

Different *superscript* in one same column shows significantly difference (P<0.05) n= 4

was caused by BLM administration continuously every day, which eventually caused chronic type lesio forming. The BLM continuous administration caused formation of progressive pulmonary fibrosis lesion (Schwaiblmair et al. 2012; Reinert et al. 2013). Increase wall thickness was caused by the existence of the fibrosis, besides it can also caused by interstitial edema (Izbicki et al. 2002).

In this study, Bleomycin toxicity was caused by low activity of BLM-*hydrolase* enzyme in the lung causing the BLM not metabolized into intoxic molecule form. Reinert et al. (2013) said that low activity of BLM-*hydrolase* enzyme in the lung resulted in an excessive apoptosis. Besides, the BLM can also stimulate endothelial cell, macrophage, and fibroblast to induce inflammatory mediator, apoptosis, and free radicals (Yamamoto & Katayama 2011). The most common symptomps from bleomycin-induced hypersensitivity pneumonitis are exertional dispnea and non-productive cough, with progressive pneumonitis dyspnea at rest, tachypnea, and cyanosis may occur (Sleijfer 2001).

Bleomycin is one of xenobiotis which able to induce radical oxygen synthesis excessively such as superoxide, hydrogen peroxide, peroxynitrite and hydroxyl radicals which is the mean mediator in inflammatory process in the lung (Oury et al. 2001). The inflammation will cause inflammatory cells entering the tissue and releasing cytokine inflammation mediator (Reinert et al. 2013). Furthermore, the cytokine caused fibroblast proliferation into myofibroblast. This inflammation and fibroblast proliferation will cause an increase of alveolar wall thickness to the pulmonary fibrosis (Izbicki et al. 2002).

Curcumin administration significantly press alveolar wall thickness incidence. The curcumin can press apoptosis and inflammation incidence; this is related to its activity as anti-oxidant and anti-inflammation (Smith et al. 2010; Bhullar et al. 2013; Basnet & Skalko-Basnet 2011). Smith et al. (2010) presented that curcumin administration significantly press pulmonary inflammation and collagen deposition in the pulmonary fibrosis induced by BLM. Punithavathi et al. (2000) said that the curcumin mediated the reduction of total number of *broncho-association limfoid follicles* (BALF) cell by preventing inflammatory cell crossing endotel and epithelial basal membrane towards the inflammation location. This is because of the curcumin stabilized the endotel and epithelial basal membrane. Therefore, the curcumin activity as anti-oxidant and anti-inflammation can press alveolar wall thickness incidence in the lung due to BLM administration.

CONCLUSION

Curcumin administration can inhibit fibrogenesis in mice pulmonary fibrosis due to the BLM application. This information can be used as first information to prevent side effect of BLM utilization to cancer patients who has to undergo chemotherapy.

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ABSTRAK

Pundir RK, Singh PK, Sadana DK. 2015. Analisis multivariate sifat-sifat morfometrik pada tiga populasi sapi asli bagian Timur Laut India yang berbeda. JITV 20(2): 79-86. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1162

Penelitian ini dilakukan untuk membedakan 3 populasi sapi bagian Timur Laut India yaitu Tripura, Mizoram, dan Maniour berdasarkan sifat-sifat morfometrik menggunakan analisis pembeda canonical untuk melihat apakah mereka sama atau berbeda. Data terdiri dari 8 sifat morfometrik yang berbeda dari 383 sapi asli Tripura (136), Mizoram (71) dan Manipur (176). Sifat morfometrik berupa panjang tubuh, tinggi bagian punggung tertinggi, lingkar jantung, lingkar tembolok, lebar wajah, panjang telinga, panjang tanduk, dan panjang ekor tanpa sendi. Semua sifat morfometrik yang diamati memiliki perbedaan yang signifikan untuk semua populasi kecuali pada panjang tanduk. Nilai semua sifat pada sapi Tripura lebih rendah dibandngkan sapi Mizoram dan Manipur. Analisis pembeda bertahap menunjukkan bahwa tinggi bagian punggung tertinggi, panjang badan, panjang telinga, panjang ekor tanpa sendi, lingkat tembolok, and panjang wajah merupakan sifat yang paling berbeda diantara ketiga populasi sapi tersebut. Jarak Mahalanobis berpasangan antara populasi sapi Tripura dan Mizoram, Tripura dan Manipur, serta Mizoram dan Manipur adalah 9,72578, 5,72089 dan 4,65239 berturut-turut dan signifikan. Dendogram menunjukkan bahwa terdapat 2 kelompok, kelompok 1 terdiri dari sapi Manipur dan Mizoram serta kelpompok 2 adalah sapi Tripura yang di[isahkan secara jelas dari kelompok 1. Penempatan individu dari populasi yang berbeda dengan pengelompokkan validasi silang mengungkapkan bahwa 84,13% sapi Tripura, 82,09% sapi Mizoram, dan 79,87% sapi Manipur ditempatkan dengan benar di dalam populasi masing-masing. Berdasarkan pengamatan ini, kita tidak dapat menyimpulkan bahwa mereka adalah tiga bangsa yang berbeda. Tetapi, informasi saat ini, pada tiga populasi sapi tersebut dapat di dimanfaatkan dalam perancangan strategi-strategi yang tepat untuk managemen dan pelestarian sapi-sapi tersebut.

Kata Kunci: Sapi Asli, Sifat Morfometrik, Analisis Multivariate, Analisis Kelompok, Analisis Pembeda Kanonikal

ABSTRACT

Pundir RK, Singh PK, Sadana DK. 2015. Multivariate analysis of morphometric traits of three different indigenous cattle populations from North East states of India. JITV 20(2): 79-86. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1162

In the present study an attempt has been made to differentiate three cattle populations of North East states of India i.e. Tripura, Mizoram and Manipur based on morphometric traits, using canonical discriminant analysis to see whether they are similar or distinct. Data consisted of eight different morphometric traits of 383 indigenous cows from Tripura (136), Mizoram (71) and Manipur (176). Morphometric traits included body length, height at withers, heart girth, paunch girth, face length, ear length, horn length and tail length without switch. All the morphometric traits under study differ significantly in these populations except horn length. All the traits, values were lower in Tripura cows than that of Mizoram and Manipur cows. The stepwise discriminant analysis showed that height at withers, body length, ear length, tail length without switch, paunch girth and face length were the most discriminating traits in these three cattle populations. The pair wise Mahalanobis distances between Tripura and Mizoram, Tripura and Manipur and Mizoram and Manipur were 9.72578, 5.72089 and 4.65239, respectively, and significant. The dendogram showed that there are two clusters; cluster one includes Manipur and Mizoram cows and cluster two Tripura cows those are clearly separated from cluster one. The Individual assignment of different cattle populations by the cross-validation classification revealed 84.13% of Tripura cows, 82.09% of Mizoram cows and 79.87% Manipur cows were assigned correctly into their respective population. Based on the present study we cannot conclude that they are three different distinct breeds. However, the present information on the three cattle populations could therefore be exploited in designing appropriate strategies for their management and conservation.

Key Words: Indigenous Cattle, Morphometric Traits, Multivariate Analysis, Cluster Analysis, Canonical Discriminant Analysis

INTRODUCTION

North East states of India comprises of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura states (Figure 1 and 2). India had 190.9 million cattle heads in the year 2012 including 151.17 million indigenous and 39.73 million exotic and crossbreds (LC 2012). All these states has around 6.9% of total cattle population of the country. The proportion of indigenous and exotic cattle as compared to whole country was 8.19% and 2.28% in these states (Table 1). In this region, there is only one registered cattle breed known as Siri (Sikkim). Rest of the cattle in this region is known as known as *Desi* (non described). There is a need to characterize these cattle populations available in these states and observed similarity/dissimilarity with existing populations using multivariate techniques. If such populations are found distinct/unique, then register them as a distinct breed and if not, search a breed/ population where they can be merged or mixed.

Previous efforts on the phenotypic characterization of breeds of livestock have been restricted to the use of analysis of variance, whereas the current trends in livestock classification involve the use of multivariate statistical tools (Traore et al. 2008; Yakubu & Akinyemi 2010; Peter et al. 2012; Aziz & Al-Hur 2013). Univariate statistical analysis analyzes each variable separately and do not explain how the populations under investigations differ when all measured morphological traits are considered simultaneously (Dossa et al. 2007). Multifactorial discriminant analyses have been found to be more suitable in assessing variation within a population and can discriminate different population types when all measured morphological traits are considered jointly. Discriminate function analysis can be used not only as a means to explain differences among populations, but also to predict group membership for sampling entities of unknown membership. Discriminate analysis has been used for differentiating populations utilizing various morphological measurements simultaneously (Herrera et al. 1996; Capote et al. 1998; Zaitoun et al. 2005; Dossa et al. 2007; Martins et al. 2009; Yakubu et al. 2010a; Yakubu et al. 2010b; Yakubu et al. 2010c; Peter et al. 2012; Aziz & Al-Hur 2013). In the present study an attempt will be made to differentiate between three cattle populations of North East states of India i.e. Tripura, Mizoram and Manipur based on morphological traits, using canonical discriminant analysis to see whether they are distinct or similar.

Table 1. Cattle population (in thousands) in north east states in India in the year 2012

State	Cattle population	Indigenous cattle	Indigenous female	Exotic cattle
Arunachal Pradesh	463.76	440.53	248.67	23.23
Assam	10307.60	9911.70	5695.29	395.90
Manipur	263.84	219.54	133.80	44.31
Meghalaya	896.00	860.75	513.61	35.25
Mizoram	34.57	23.28	14.75	11.30
Nagaland	234.97	106.02	64.46	128.95
Sikkim	140.47	13.95	8.90	126.52
Tripura	948.79	815.69	502.89	133.31
Total	13290.00 (6.96%)	12391.46 (8.19%)	7182.37 (8.05%)	898.77 (2.28%)
India	190904.00	151172.00	89223.00	39731.00

Source: 19th LC 2012 (http://dahd.nic.in/dahd/WriteReadData/Livestock.pdf)

Figure 1. North East zone of India
Source: http://www.mapsofindia.com/states/

Figure 2. North East zone of India
Source: http://www.mapsofindia.com/states/

MATERIALS AND METHODS

Collection of data and location of study

Data consisted of 8 different morphometric traits of 383 indigenous cows from Tripura (136 from West, South, Gomti and Dhalai districts), Mizoram (71 from Champhai and Kolasib districts) and Manipur states (176 from Imphal East, Imphal west and Churachandpur districts) of the union of India. These indigenous cattle in all the three states were not described earlier and so far known as nondescript/desi. All the measurements were recorded by the same recorder to avoid between recorder effects. All the traits were recorded from the left side of the cows. The circumference measurements were taken from a tape while the other measures were taken by a measuring stick. Cows were reared through the extensive management system and originated from different herds in different states.

Measured traits

The recorded morphometric traits were body length (the distance from the point of the shoulder joint to the point of the pin bone), height at withers (the distance from the highest point of withers to the ground), heart girth (the circumference of the chest just behind the elbow joint), paunch girth (the circumference at paunch region just anterior to the hip joint), ear length (distance from the point of attachment of ear to the tip of the ear) face length(distance from between the horn site to the lower lip), horn length (distance from part of horn attachment to the tip of the horn) and tail length without switch (measured from the root of tail droop to the tip of the tail excluding switch). Physical traits like coat colour, body shape, face, horns, udder and tail characters were also recorded.

Statistical analysis

Means, standard errors and coefficients of variation of the different morphometric traits were calculated using General linear model PROC GLM (SAS 2009) with state effect. The DUNCAN's multiple range test was performed by all the means of different morphometric traits to see whether states are differ significantly or not. Stepwise discriminate procedure (SAS 2009) was applied using PROC STEPDISC to determine which morphological traits have more discriminant power than others. The relative importance of the morphometric variables in discriminating between the cattle populations was assessed using the level of significance, partial R2 and F-statistic. The CANDISC (SAS 2009) procedure was used to perform univariate and multivariate one-way analysis that

calculated the Mahalanobis distances between the three cattle populations. Based on the Mahalanobis distance matrix dendogram was created using PROC CLUSTER (SAS 2009) with Average Linkage Method. The ability of these canonical functions to assign each individual animal to its respective population calculated as the percentage of correct assignment to each cattle population using the DISCRIM (SAS 2009) procedure by Nearest Neighbour Discriminant Analysis. The cross-validation approach was used for assignment of individual to their respective population in which one individual is removed from the original matrix and the discriminant analysis is then performed from the remaining observations and used to classify the omitted individual. It also providesan unbiased estimate of error. The proportion of individuals correctly re-allocated is taken as a measure of the morphological distinctness of the population.

RESULTS AND DISCUSSIONS

Tripura, Mizoram and Manipur states are adjoining and located in eastern part of the country. In these states temperature ranged from 10°C to 32ºC. Rice is major crop and no green fodder was grown for animals. Annual rainfall is high more than 2000 mm. Animals were reared mainly on extensive system of management i.e. grazing from morning to evening. Physical traits recorded on these three cattle populations did not reveal significant differences as majority of traits were over lapping. Analysis of physical traits (frequencies) in these cattle population showed that they are differing in proportion of different physical traits, but there was not a single physical trait which can differentiate them strictly. In general animals were small in size with the cylindrical type of body. Animals were well built and compact with strong legs. The coat colour varied in different colours i.e. brown, black and grey/white but brown colour predominates. Dewlap and hump were small. The head was small. Face was short and concave. Ears were small to moderate in length and horizontal in orientation. The neck was short in length and thin. Horns were small, black or gray in colour. Orientation was outward and then upward. Hoofs were black. Muzzles were brown and black. Udder was small, not well developed and milk veins were not prominent. Sizes of fore and rear udder were small. Teats were small 5-12 cm long. Penis sheath flap was short and tucked up with the body. The tail was longer up to the hock with black, brown and white switch. Temperament was docile in all the cases. Cows of these three cattle populations are presented in Figures 3-5.

Descriptive statistics of the morphological traits of three different indigenous cows from three different states are given in Table 2. All the traits under study differ significantly in these populations except horn length. All the traits, values were lower in Tripura cows than that of Mizoram and Manipur cows. Manipuri and Mizoram cows differ significantly in body length, ear length and tail length without switch.

The considerable variation in body dimensions of the three cattle populations might not be unconnected with individual population potential and peculiarities. The minimum and maximum variability was observed in horn length and ear length, respectively. The estimates of body length obtained in the present study were in agreement with the reports of Pundir et al. (2013) in Uttara cows, Pundir et al. (2012) in Pithoragarh cows and Pundir et al. (2009) in Bargur cows. However, higher estimates of body length were observed by Singh et al. (2012) in Pullikumam cows, Pundir et al. (2011) in Kankrej cows and Pundiret al. (2007) in Kenkatha cows.The estimates of height at wither, heart girth and paunch girth were comparablewith the reports of Pundir et al. (2012; 2013). Higher estimates of height at wither were reported by Singh et al. (2012), Pundir et al. (2007; 2011).

Figure 3. Indigenous cattle of Tripura

Figure 4. Indigenous cattle of Manipur

Figure 5. Indigenous cattle of Mizoram

Table 2. Descriptive statistics of different morphometric traits (cm) in indigenous cows of NEH states

State	Overall (383)		Tripura (136)		Mizoram (71)		Manipur (176)	
Trait	Mean+S.E.	C.V.	Mean+S.E.	C.V.	Mean+S.E.	C.V.	Mean+S.E.	C.V.
Body lenght	101.14+0.46**	8.75	98.09+0.54^{c}	6.43	109.03+1.39^{a}	10.77	100.32+0.59^{b}	7.88
Height at wither	101.80+0.45**	8.81	93.39+0.44^{b}	5.52	106.92+0.84^{a}	6.67	106.22+0.51^{a}	6.48
Heart girth	132.45+0.72**	10.72	122.05+1.09^{b}	10.46	139.52+1.59^{a}	9.67	137.69+0.78^{a}	7.53
Paunch girth	136.89+0.82**	11.67	125.41+1.08^{b}	10.13	146.64+1.80^{a}	10.41	142.12+0.98^{a}	9.10
Ear length	19.26+0.13**	13.70	19.47+0.24^{b}	14.38	18.02+0.33^{a}	15.88	19.59+0.17^{b}	11.58
Face length	36.73+0.15**	8.63	35.30+0.20^{b}	6.71	36.15+0.37^{a}	8.82	38.06+0.23^{c}	8.02
Tail length without switch	71.20+0.38**	10.57	68.63+0.51^{a}	8.88	68.54+1.07^{a}	13.23	74.31+0.50^{b}	8.96
Horn length	11.34+0.26	4.37	10.87+0.50	5.20	11.01+0.42	31.90	11.85+0.38	41.01

Table 3. Summary of step wise selection of different traits in indigenous cows of of NEH states

Variable Entered	Partial R-Square	F Value	Pr>F	Wilks' Lambda	Pr< Lambda	Average Squared Canonical Correlation	Pr> ASCC
Height at wither	0.5114	180.06	<0.0001	0.488	<0.0001	0.255	<0.0001
Body length	0.1315	25.96	<0.0001	0.423	<0.0001	0.320	<0.0001
Eal length	0.1814	37.90	<0.0001	0.347	<0.0001	0.390	<0.0001
Tail length without switch	0.1112	21.33	<0.0001	0.308	<0.0001	0.432	<0.0001
Paunch girth	0.0852	15.84	<0.0001	0.282	<0.0001	0.451	<0.0001
Face length	0.0770	14.14	<0.0001	0.260	<0.0001	0.477	<0.0001

Similar estimation of face length and tail length without switch were obtained by Pundir et al. (2007; 2012; 2013). Higher estimates of both the traits were observed by Singh et al. (2012), Pundir et al. (2009; 2011). Comparable estimates of horn length to the present study was reported by Pundir et al. (2013) However, Pundir et al. (2007; 2009; 2011; 2012) and Singh et al. (2012) obtained higher estimates of the same trait.

The stepwise discriminate analysis showed that height at wither, body length, ear length, tail length without switch, paunch girth and face were the most discriminating variables between these three cattle populations (Table 3). Their respective partial R2 were 0.5114, 0.1315, 0.1814, 0.1112, 0.0852 and 0.0770, respectively, with high significant values (P<0.0001). The corresponding F values for these traits were 180.6, 25.96, 37.90, 21.33, 15.84 and 14.14, respectively and highly significant.

These six morphological variables obtained in the present study are more important and informative, and could be used to assign the three cattle populations into distinct populations, thereby reducing the errors of selection in future breeding and selection programmes. Similar to the present study, Yakubu et al. (2010a) also reported height at wither and face length most discriminating traits in two distinct cattle breeds. In an attempt to distinguish between brown and gray Bengal goats, Mukeherjee et al. (1979) reported significant differences between both breeds due to body length and chest circumference.

Herrera et al. (1996) employed discriminate analysis on several body measurements such as, shin circumference, chest girth, chest depth, rump length and width, and shoulder height to differentiate among five Spanish goat breeds. Zaitoun et al. (2005) applied discriminant analysis on 20 metrical variables to discriminate among different goat genetic groups.

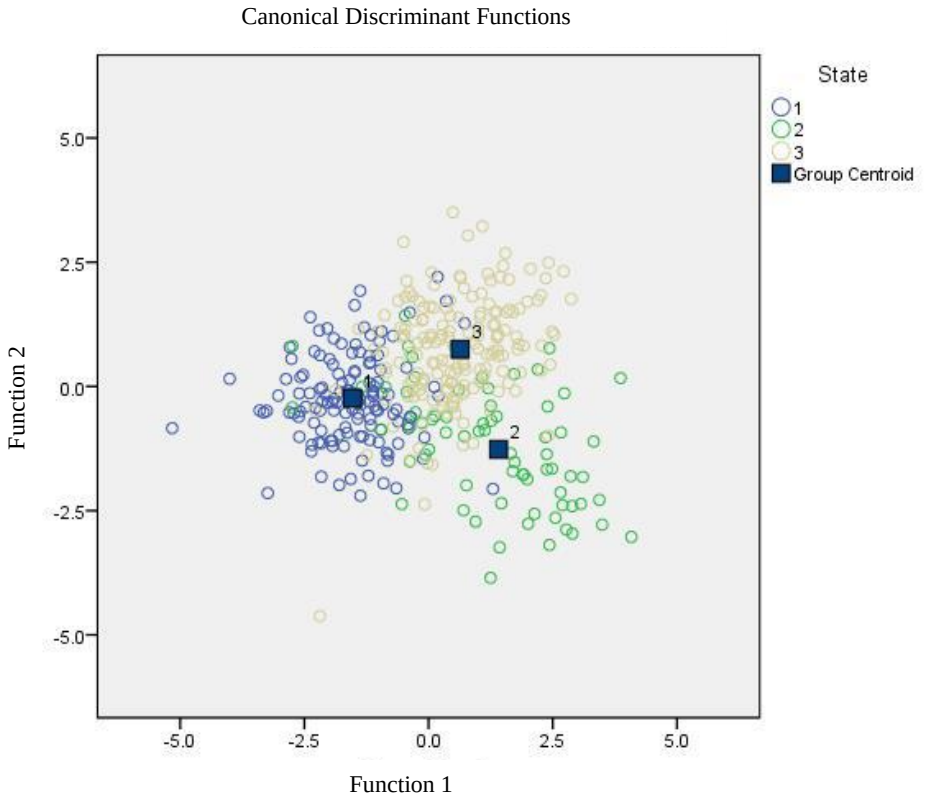

Figure 6. Canonical discriminiant functions of three indigenous cows populations (State 1 Tripura 2 Mizoram 3 Manipur)

In these studies, step-wise discriminant analysis was first applied to select the most important discriminator variables used for differentiation among breeds under study. The canonical discriminant function representation is shown in Figure 6 which revealed overlapping of these populations in the present study.The Mahalanobis distances between three cattle populations are given in Table 4. The pairwise distance Tripura and Mizoram, Tripura and Manipur and Mizoram and Manipur were 9.72578, 5.72089 and 4.65239, respectively, and highly significant (P<0.0001). Yakubu et al. (2010a) Observed Mahalanobis distance between the two cattle populations as 7.19 which was high and significant and indicated that that they belong to genetically different groups. Yakubu et al. (2010c) estimated Mahalanobis distance of 72.28 between West African Dwarf and Red Sokoto goats in Nigeria, indicating that there is considerable genetic variation between both breeds. Aziz & Al-Haur (2013) observed Mahalanobis distance of 0.55 between two lines of goat and between Ardi and each of Line1 and Line2 were 25.03 and 21.45, respectively.

The dendogram (Figure 7) based on the average linkage methodshowed that there are two clusters; cluster one includes Manipur and Mizoram cows and cluster two Tripura cows those are clearly separated from cluster one.

Table 4. Mahalanobis distances between three different populations of indigenous cows in North East States

State	Tripura	Mizoram	Manipur
Tripura	0	9.72578	5.72089
Mizoram	P<0.0001	0	4.65239
Manipur	P<0.0001	P<0.0001	0

In the lower triangular probability of significance is shown

The individual assignment to different cattle populations by the Cross-validation classification was given in table 5. The proportion of individuals correctly assigned to their respective population is considered as a measure of the morphological distinctness of the population.High values of error 0.158, 0.179 and 0.201 were observed for Tripura, Mizoram and Manipur cattle populations, respectively. The reason for this misclassification may be a high degree of intermingling these populations as they are from the adjoining states. The high morphological distances between the cattle populations coupled with high correct assignment to source populations is an indication that they belong to different populations. But there was no distinct physical trait which could differentiate these populations.

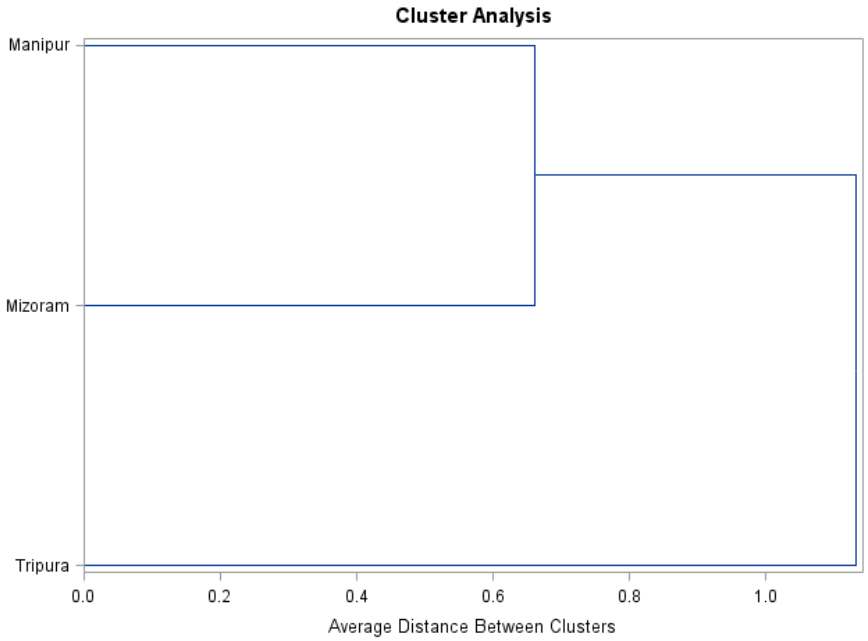

Figure 7. Dendogram showing similarity/distinctness in three indigenous cows populations

Table 5. Percent of individual cows classified in to different populations of north east states

Population	Tripura	Mizoram	Manipur	Total
Tripura	106	7	13	126
	(84.13)	(5.56)	(10.32)	(100)
Mizoram	4	55	8	67
	(5.97)	(82.09)	(11.94)	(100)
Manipur	13	18	123	154
	(8.44)	(11.69)	(79.87)	(100)
Error level	0.158	0.179	0.201	0.158
Priors	0.333	0.333	0.333	

Yakubu et al. (2010a) reported that 85.48% of Bunaji cattle and 96.55% of Sokoto Gudali classified into their source population assigned correctly by the Nearest Neighbour Discriminant Analysis. Aziz & Al-Haur (2013) observed 100% assignment of Ardi animals into their genetic group and percentages of animals assigned in Line1 and line2 were 86.10 and 42.55, respectively. The use of multivariate discriminant analyses therefore could be successfully used in morphometric differentiation. Similar reports on goats (Dossa et al. 2007; Yakubu et al. 2010a; Yakubu et al. 2010b; Yakubu et al. 2010c), sheep (Traore et al. 2008; Yakubu & Akinyemi 2010), cattle (Ndumu et al. 2008) and buffalo (Johari et al. 2009) respectively were observed.

CONCLUSION

In the present study correct assignment of individual animals to their respective population ranged from 80 to 84% but we could not get a physical /discontinuous trait which can distinct these populations may be due to intermingling. Canonical discriminiantanalysis also showed that these three indigenous cow populations were overlapping, so we cannot conclude that they are distinct breeds.

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Isolation and Identification of Indigenous Lactic Acid Bacteria from North Sumatra River Buffalo Milk

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ABSTRAK

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Susu kerbau merupakan salah satu sumber bakteri asam laktat (BAL) yang berpotensi sebagai kultur untuk starter maupun probiotik. Penelitian ini bertujuan untuk mengisolasi dan mengidentifikasi BAL asal susu kerbau sungai Sumatera Utara, serta menguji ketahanannya pada pH rendah. Bakteri asam laktat diisolasi dan ditumbuhkan pada medium *De Man Rogosa Sharpe Agar* (MRSA). Isolasi dilakukan sampai diperoleh kultur murni. Identifikasi BAL dengan pengujian karakteristik morfologi, fisiologi dan biokimia. Karakteristik morfologi dilakukan dengan pewarnaan Gram dan melihat bentuk sel. Karakteristik fisiologi dilakukan dengan uji ketahanan pada pH 4,5 dan suhu 45^{o}C. Karakteristik biokimia dilakukan dengan uji produksi CO_2, NH_3 dan dekstran. Pengujian sifat probiotik dilakukan dengan menguji ketahanan isolat BAL pada pH rendah. Pengujian dengan kit API tes CHL 50 untuk mengetahui jenis spesies dari sepuluh BAL terpilih. Hasil identifikasi diperoleh 41 isolat BAL, yang secara umum memiliki karakterisitik Gram positif, katalase negatif, berbentuk batang dan bulat. Pengujian ketahanan BAL pada pH rendah (pH 2) menunjukkan penurunan jumlah populasi sebanyak 1,24±0,68 log cfu/ml. Isolat terbaik dari pengujian ketahanan pada pH rendah adalah isolat L12, L16, L17, L19, L20, M10, P8, S3, S19 dan S20. Hasil pengujian dengan API test CHL 50 pada sepuluh isolat terpilih tersebut menunjukkan ada empat spesies BAL dari susu kerbau sungai Sumatera Utara, yaitu *Lactobacillus plantarum, L. brevis, L. pentosus* dan *Lactococus lactis*.

Kata Kunci: Susu Kerbau, BAL, Isolasi, Identifikasi

ABSTRACT

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Buffalo milk is a source of various lactic acid bacteria (LAB) which is potential as culture starter as well as the probiotic. This study was conducted to isolate and identify LAB from indigenous North Sumatra river buffalo milk. Lactic acid bacteria was isolated and grown in medium De Man Rogosa Sharpe Agar (MRSA). The isolation was conducted to obtain pure isolate. The identification of LAB was studied in terms of morphology, physiology, biochemistry and survival on low pH. Morphology tests were conducted by Gram staining and cell forming; physiology tests were conducted for growing viability at pH 4.5 and temperature at 45^{o}C; whereas biochemistry tests were conducted for CO_2, dextran and NH_3 productions. Determination of LAB species was conducted using Analytical Profile Index (API) test CHL 50. Results of identification showed that 41 isolates were identified as LAB with Gram-positive, catalase-negative, rod and round shaped characteristics. Resistance test done to low pH (pH 2) for the lactic acid bacteria showed decrease of bacteria viability up to1.24±0.68 log cfu/ml. The resistant isolates at low pH were L12, L16, L17, L19, L20, M10, P8, S3, S19 and S20. Identification with API test CHL 50 for 10 isolates showed that four isolates were identified as *Lactobacillus plantarum, L. brevis, L. pentosus* and *Lactococuslactis*.

Key Words: Buffalo Milk, LAB, Isolation, Identification

INTRODUCTION

Milk is one of food stuffs that naturally containing Lactic Acid Bacteria (LAB) which is generally used for culture starter forming various processed food products. The LAB originated from milk is also potentially developed into probiotic for functional food development. Probiotic was life bacteria, given as food and feed supplement which have beneficial effects for health of both human and animals by improving intestinal microflora balance (Roberfroid 2000). Study of LAB's potential as probiotic of indigenous materials are continuously done, especially to be used as functional food.

LAB isolation of various milk had been done, among others from raw cow milk (Abdullah & Osman 2010), goat milk (Tserovska et al. 2002; Setyawardani et al. 2013), Bima horse milk (Antara et al. 2009), wild horse milk (Sugitha et al. 2011), Sumbawa horse milk (Sujaya et al. 2008), sheep milk (Iranmanesh et al. 2012), camel milk (Ahmed et al. 2002; Khedid et al. 2009; Abbas & Mahasneh 2015), and breast milk (Nuraida et al. 2011). LAB isolation of fresh buffalo milk was done in India, Pakistan and Egypt both of fresh buffalo milk or produced buffalo milk products (Aziz et al. 2009; Tambekar et al. 2009; Singh & Sharma 2009; Patel & Patel 2012; Sharma et al. 2013; Shafakatullah & Chandra 2014; Kumar et al. 2014). In Indonesia, LAB isolation from the produced buffalo milk products had been done to produce a product of West Sumatran buffalo called "dadih" (Surono 2003; Sunaryanto & Marwoto 2013), whereas isolation and identification of LAB from fresh buffalo milk in Indonesia has not been much done.

North Sumatra river buffalo is one of genetic sources of Indonesian local animal which has not been explored. River buffalo milk production could reach 8 liter/day. Its quality was better compared to cow milk, where in the content of protein and fat was higher but its cholesterol content was lower (Damayanthi et al. 2014). Exploration of LAB from North Sumatra river buffalo and its utilization to produce the culture starter of probiotic product currently have not been much reported. This LAB exploration was first step to get probiotic bacteria candidate by testing several probiotic characteristics (Rizqiati et al. 2015); in further it was applied in mozzarella cheese into probiotic mozzarella cheese with North Sumatra river buffalo milk as raw material.

One condition for microorganism called as probiotic was its capability to survive in intestinal track condition such as endurance to low pH and bile salt (Nuraida et al. 2011). The first exposure of probiotic when entering the intestinal track was a gastric with pH around 2. Endurance test to low pH was needed to determine whether the LAB culture was survive in the gastric acid or not (Wildman & Medeiros 2000).

This study was aimed to isolate the LAB from North Sumatra river buffalo milk and identify it based on morphology, physiology, biochemical, and identify species of the LAB. Besides, this study was aimed to select LAB isolate which could survive in low pH (pH 2) which was one of probiotic characteristic tests. Isolation and identification results of LAB became first step of selection of LAB from North Sumatra river buffalo milk as a probiotic.

MATERIALS AND METHODS

Materials used in this study were 24 samples of river buffalo milk from 4 locations of buffalo farming in North Sumatra, namely: Lubuk Pakam, Medan, Patumbak, and Siborong-borong where from each location was taken 6 samples. The milk samples were brought in cold sterile tube in the cooling box to the Integrated Laboratory of Faculty of Animal Science of Bogor Agricultural University. Conducted tests were isolation and identification of LAB from the river buffalo milk. The isolation was done using media De Man Rogosa Sharpe (MRS). LAB identification consisted of characteristic of morphology, physiology, and biochemical. Characteristic tests of the probiotic were done by testing LAB resistance to low pH. LAB identification by API test CHL 50 was done to determine its species.

LAB isolation

LAB isolation technique referred to Khedid et al. (2009) which was modified. The buffalo milk samples (10 ml) was taken aseptically and inserted into 90 ml of sterile physiological NaCl solution (0.85%), and further it was done a gradual dilution into 10^5 dilutions. One ml diluted sample was distributed on MRS agar (MRSA) media containing of bromo cresol purple (BCP) 0.01% on petridish, and then the samples were incubated anaerobically for 24 hours at 37^{o}C. LAB colony seemed as colony which was surrounded by yellow zone, and further that colony was taken and scratched on MRSA media. That scratching was done continuously to obtain one pure colony.

Morphology characteristic to identify the LAB

Characterizing of cell morphology was aimed to see isolate form and Gram staining characteristic. Cell form was viewed by microscope and done after the Gram staining. Cell morphology expected was positive Gram with tube or oval cell (Iranmanesh et al. 2012).

Physiology characteristic to identify the LAB

Physiology characteristic test to identify the LAB consisted of resistance test to temperature and pH. Resistance test to temperature was done to select LAB which could still grow at 45^{o}C and 37^{o}C for 2-5 days as control. Resistance test to pH was aimed to select LAB isolate which could still grow at MRSB media with acid

environment with pH of 4.5 and in neutral condition (pH 7) as control, and further were incubated at 37ºC for 7 days. Growth was marked by turbidity in the media (Aziz et al. 2009).

Biochemical characteristic to identify the LAB

Biochemical characteristic test to identify the LAB consisted of catalase test, production test of CO_2, dextran, and NH_3. CO_2 production test was conducted to determine isolate capability to produce CO_2 from glucose, which showed whether the isolate was homofermentative or heterofermentative. Catalase test was done to determine the isolate capability to produce catalase enzyme. Catalase test was done using hydrogenperoxide (H_2O_2) 3%. Dextran production test was done to determine whether the isolate could produce dextran (mucus formed) which was generally produced by *Leuconostoc* genus. NH_3 production test was done to determine isolate which could produce ammoniac which was generally produced by *Streptococcus* genus (Harrigan 1998).

Probiotic characteristic testing of LAB resistance in low pH

LAB resistance test to low pH was done using PBS media by regulating the pH into 2 using HCL 0.1 N and incubated for 3 hours at 27ºC. LAB number was counted before and after the incubation. Cell number was counted by casting method using MRSA. Each test was done 3 times duple repetition (Lin et al. 2006).

LAB identification using kit API CHL 50

LAB identification using kit API CHL 50 (Biomerieux, France) was conducted to determine species of the LAB. The testing was done by 1 ose of LAB inserted into 10 ml MRSB media then incubated at 37ºC for 24 hours. LAB culture was centrifuged 9800 x g for 10 minutes. Separated pellet was inserted into API 50 CHL media by sterile pipette and homogenized. The culture inserted into 50 pits of kit API CHL 50 strips. All of the pits were closed by paraffin oil to give anaerobic environment and incubated at 37ºC for 24-48 hours. Testing parameter was color change from blue into yellow after incubation for 24-48 hours because of formation of acid which was detected by pH change. Observation result was processed by software Apiweb™ (Gawad et al. 2010).

RESULT AND DISCUSSION

LAB isolates

LAB isolation used MRSA media, added by BCP as bacteria indicator of acid former, where the isolate which produced acid, would form yellow zone. LAB colony appearance was in form of white oval surrounded by yellow zone. It was obtained 96 isolates from North Sumatra river buffalo milk from 4 locations, namely: Lubuk Pakam (L), Medan (M), Patumba (P), and Siborong-borong (S), where each location was obtained successfully 24 isolates. LAB colony grew in the MRSA media, which was added by BCP, would seem as colony surrounded by yellow zone (Surono 2004).

LAB identification

Morphology characteristic of LAB isolate was identified through Gram staining. It showed that from 96 pure isolates which was isolated successfully from river buffalo milk, 84 isolates were positive Gram bacteria and 12 isolates were negative Gram bacteria. Test result of LAB cell form showed 19 of the 84 isolates were in oval form (22.6%) and 65 isolates were in tube form (77.4%). Lactic Acid Bacteria was in oval or tube form positive Gram bacteria, did not form spore, able to ferment carbohydrate, negative catalase, and microaerophilic (Axel's son 2004). In the testing of morphology characteristic from 96 isolates, 84 isolates were of positive Gram bacteria. It was followed by physiology characterizing.

Physiology characteristic testing consisted of resistance to temperature and pH. It showed 78 of 84 isolates (92.8%) were resistant to high temperature and 6 isolates (7.1%) were not resistant to high temperature. Surono (2004) said that one of factors affected bacteria's growth was temperature. Elgadi et al. (2008) said that resistance of 14 isolates from fresh milk could grow at 45ºC of temperature. According to El Soda et al. (2003), termophylic *lactobacilli* and *cocci* groups could grow at 45ºC of temperature, but could not at 10ºC of temperature. Mesophylic group could not grow at 45ºC but could grow at 10ºC of temperature. Mesophilic *lactococci* could grow at 10ºC but could not grow at 45ºC of temperature. *Enterococci* group could grow at 45ºC and 10ºC of temperature.

Resistance test to pH resulted in 70 isolates (89.7%) of 78 could grow at 4.5 of pH and 8 isolates (10.3%) could not grow at that pH. According to Fowoyo & Ogunbanwo (2010), isolate type of lactic acid which

lived at 2 of pH was fewer. This type of bacteria had better ability to life in very acidic condition, and able to produce bigger organic acid number. Organic acid produced could be used to improve aroma, texture, and flavor of fermented products. In the physiology characteristic testing, 70 of 84 isolates were resistant to high temperature and low pH. It was followed by biochemistry characteristic testing.

Result of biochemistry characteristic testing showed that in testing of production of CO_2 from glucose to 70 isolates of indigenous LAB of North Sumatra river buffalo milk showed that 21 isolates (30%) could produce CO_2 (heterofermentative), whereas 49 isolates (70%) did not produce CO_2 (homofermentative). Abdulah & Oman (2010) also reported similar result that homofermentative LAB number was found more in cow milk, cheese, and fermented milk. According to Axelsson (2004), based on fermentation pattern, LAB was divided into 3 groups, namely: homofermentative obligative, heterofermentative facultative, and heterofermentative obligate LAB.

Catalase testing showed that 52 of 70 isolates (74.3%) did not produce O_2 vesicle, so that it was grouped into negative catalase bacteria, whereas 18 isolates (25.7%) produced O_2 vesicle and grouped into positive catalase bacteria. Catalase testing was aimed to determine catalase enzyme presence in the culture of bacteria starter. Catalase enzyme production could be known by dripping H_2O_2 on bacteria preparate. If there was gas vesicle, it showed that the bacteria released O_2 and grouped into positive catalase bacteria. Bacteria that did not release the O_2 vesicle showed that the bacteria had peroxidase enzyme which could prevent O_2 production and it was stated as negative catalase bacteria (Surono 2004).

Testing result of production of dextran from sucrose to 52 isolates of indigenous LAB of North Sumatra river buffalo milk showed that 4 isolates (7.7%) produced dextran positively, whereas 48 isolates (92.3%) did not produce dextran. It was concluded that the LAB isolates which did not produce dextran did not included into *Leuconostoc* group. One of characters of the *Leuconostoc* is dextran production visualized as mucoid. Dextran was defined as water soluble polygosacaride formed from α1-6 glucosides with proportion of 0-20% (Sarwat et al. 2008).

Testing of 48 isolates in producing NH_3 from arginine showed that isolates (14.6%) produced NH_3 positively, whereas 41 isolates (85.4%) did not produce NH_3. Isolates that produced NH_3 did not selected to further testing because by producing NH_3, it was feared will affect the product aroma. Tserovska et al. (2002) said that 60% of LAB from cheese and milk able to produce NH_3 from arginine.

Research result of LAB identification by biochemistry characteristic testing showed that it was obtained 40 of 70 LAB isolates which meet LAB characteristics, so that it was continued to further test. The further test was probiotic characterizing to evaluate LAB resistance to low pH. LAB 41st characteristic identified successfully was presented in Table 1.

LAB resistance testing of low pH

LAB isolates resistance to low pH was one of probiotic conditions, because stress of probiotic LAB was started at the time the LAB exposured by acidity condition of the gastric. According to Wildman & Medeiros (2000), gastric had around 2 of pH. Resistance testing of low pH was needed to determine capability of LAB culture of isolates of river buffalo milk to survive to the gastric acidity.

Figure 1, shows LAB population before and after exposured to low pH which experienced a population decline of 1.24±0.68 log cfu/ml. In other study, in LAB isolate from buffalo milk in India showed just 2 of 3 isolates tested that survived at 2 of pH for 3 hours incubation, whereas 1 isolate only survived for 1.5 hours (Shafakatullah & Chandra 2014). In the testing of LAB isolate from breast milk, all of the isolates experienced number cell decreasing with different decreasing value to each isolate with decreasing range around 0.57-7.24 log cfu/ml (Nuraida et al. 2011). Resistance testing at low pH of LAB isolate from goat milk showed that all of the tested isolates experienced population decrease less than 1 log cfu/ml (Setyawardani et al. 2014).

Ability to survive in better acidic condition for each isolate was showed by different cell number change of all tested isolates. According to Nuraida (2011), isolate ability to survive at this acidic condition was *strain dependent*. This was due to cytoplasm membrane difference of each bacteria. The difference affected characteristic and permeability of membrane.

Under acidic condition, LAB could maintain acidity of cytoplasm, so that protein and enzyme in the cell could still work optimally. LAB isolate was adaptable in low pH because it had an internal cell pH regulation. This can be achieved by existence of enzyme synthesis and producing proton (H+) from inside of the cell that was occurred through ATP hydrolysis (H+-ATPase) process. The Lactic Acid Bacteria was survive from acid damage because the existence of histidine decarboxylase and arginine of deaminase enzyme. LAB tolerance of high acidity was caused by its capability to maintain more alkaline pH of cytoplasm than extracellular pH (Pan et al. 2011).

Result showed of North Sumatra river buffalo milk that had the best resistance or experienced lowest population decrease were isolate L12, L16, L17, L19, L20, M10, P8, S3, S19, and S20. The 10 of that isolates were selected to be tested of LAB species using API test CHL 50.

Table 1. Identification of LAB isolates from North Sumatra river buffalo milk

Isolate Code	Catalase	Gram	Cell Form	pH 4,5	Temperature (45ºC)	CO_2 Production.	NH_3 Production	Dextran Production
L7	-	+	tube	+	+	+	-	-
L8	-	+	tube	+	+	-	-	-
L11	-	+	tube	+	+	+	-	-
L12	-	+	tube	+	+	-	-	-
L13	-	+	tube	+	+	-	-	-
L14	-	+	oval	+	+	+	-	-
L15	-	+	oval	+	+	-	-	-
L16	-	+	tube	+	+	+	-	-
L17	-	+	tube	+	+	+	-	-
L18	-	+	oval	+	+	+	-	-
L19	-	+	tube	+	+	+	-	-
L20	-	+	tube	+	+	-	-	-
M1	-	+	tube	+	+	-	-	-
M2	-	+	oval	+	+	+	-	-
M3	-	+	tube	+	+	-	-	-
M10	-	+	tube	+	+	-	-	-
M11	-	+	tube	+	+	-	-	-
M12	-	+	oval	+	+	+	-	-
M14	-	+	oval	+	+	-	-	-
P1	-	+	tube	+	+	+	-	-
P2	-	+	tube	+	+	-	-	-
P4	-	+	tube	+	+	-	-	-
P5	-	+	tube	+	+	+	-	-
P6	-	+	tube	+	+	-	-	-
P7	-	+	tube	+	+	+	-	-
P8	-	+	oval	+	+	-	-	-
P9	-	+	tube	+	+	+	-	-
P10	-	+	tube	+	+	+	-	-
P11	-	+	tube	+	+	-	-	-
P13	-	+	tube	+	+	-	-	-
P14	-	+	oval	+	+	+	-	-
S3	-	+	tube	+	+	-	-	-
S6	-	+	tube	+	+	+	-	-
S9	-	+	tube	+	+	-	-	-
S10	-	+	tube	+	+	-	-	-
S11	-	+	tube	+	+	+	-	-
S12	-	+	tube	+	+	+	-	-
S13	-	+	oval	+	+	-	-	-
S14	-	+	oval	+	+	+	-	-
S19	-	+	oval	+	+	-	-	-
S20	-	+	tube	+	+	-	-	-

L = Lubuk Pakam
M = Medan
P = Patumbak
S = Siborong-borong
\+ = There is a growth
\- = There is no growth

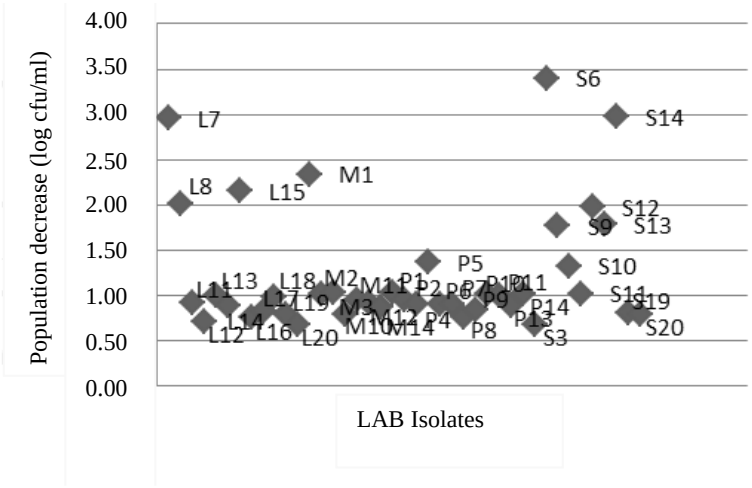

Figure 1. Population decrease of LAB after it exposured by low pH

Table 2. Identification of LAB species using API test CHL 50

Code of isolate	Name of LAB species	Percentation (%)
L12	*Lactobacillus pentosus*	94.4
L16	*Lactobacillus brevis*	96.6
L17	*Lactobacillus brevis*	96.6
L19	*Lactobacillus brevis*	99.5
L20	*Lactobacillus plantarum*	99.9
M10	*Lactobacillus plantarum*	99.9
P8	*Lactococcus lactis*	96.2
S3	*Lactobacillus plantarum*	99.9
S19	*Lactococcus lactis*	96.2
S20	*Lactobacillus plantarum*	98.3

L= Lubuk Pakam; M= Medan; P= Patumbang; S= Siborong-borong

LAB identification using API test CHL 50

Identification of LAB species using API test CHL 50 was done to the best 10 isolates based on resistance testing to low pH. It was presented completely in Table 2. Testing by API test CHL 50 resulted in 4 LAB species, namely: *Lactobacillus plantarum* (4 isolates), *Lactobacillus brevis* (3 isolates), *Lactobacillus pentosus* (1 isolate) and *Lactococus lactis* (2 isolates).

L12 isolate was identified as *Lactobacillus pentosus* with acid level of 94.4%, isolate L16, L17, and L19 were identified as *L. brevis* with acid level of 96.6%; 96.60%, and 99.50%, respectively. Isolate L20, M10, S3, and S2 were identified as *L. plantarum* with same acid level of 96.20%. Based on genotyping testing result by analysis of gene 16 sequences of S rRNA and after alignment of nucleotide sequence from selected isolate (isolate S3) on GenBank database showed that S3 isolate had similarity to the isolates of *L. plantarum* strain JCM 1149 with access code NCBI BLAST NR. 117813.1 with similarity level of 99% (unpublished data). This result showed that isolation and identification of API test CHL 50 was in line with the genotyping sequence of gene 16 S rRNA.

Testing result of species identification using API test CHL 50 of isolates from buffalo milk in India showed that there were 6 LAB species, namely: *L. bulgaricus, L. plantarum, L. lactis, L. acidophilus, L. brevis*and *L. rhamnosus* (Tambekar et al. 2009; Singh & Sharma 2009; Azis et al. 2009; Syafakatullah et al. 2014). LAB species testing of processed buffalo milk (cruds) in Indonesia showed that there were 6 LAB species, namely: *L. brevis, L. plantarum, L. casei, L. paracasei, Lactococcus lactis, Leuconostoc mesenteroides* (Surono 2003), whereas Sunaryanto & Mawoto (2012) identified successfully one of LAB species, namely: *L. plantarum*.

CONCLUSION

In this research 41 LAB isolates from North Sumatra river buffalo milk were isolated and identified. They survived successfully in low pH. Identification result of species tested by API test CHL 50 showed that from selected 10 isolates, 4 species were identified successfully, namely: *Lactobacillus plantarum, L. brevis, L. pentosus* and *Lactococus lactis.*

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Effectivity of Probiotic, Micromineral Enriched Yeast and Their Combination with *Azadirachta indica* Leaves Containing Tannin on Fermentability and Digestibility of *Pennisetum* hybrid

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ABSTRAK

Sofyan A, Sakti AA, Karimy MF, Julendra H, Istiqomah L, Herdian H, Damayanti E, Suryani AE. 2014. Efektivitas probiotik, mikromineral terkorporasi khamir yang dikombinasikan dengan daun *Azadirachta indica* mengandung tanin terhadap fermentabilitas dan kecernaan *Pennisetum* hybrid. JITV 20(2): 95-104. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1164

Eksplorasi imbuhan pakan organik untuk ternak saat ini terus dilakukan untuk menggantikan antibiotik sebagai pemacu pertumbuhan. Probiotik dari bakteri asam laktat telah digunakan secara luas untuk mendukung keseimbangan mikroba saluran pencernaan. Selain itu, mineral organik ditambahkan pada pakan untuk meningkatkan ketersediaan dalam mencegah kelainan metabolisme akibat defisiensi mineral. Penelitian ini bertujuan untuk mengevaluasi penambahan probiotik (*Pediococcus acidilactici* RS2) dan mikromineral terkorporasi khamir/*micromineral enriched yeast* (MEY) yang dikombinasikan dengan tanin dari daun mimba (*Azadirachta indica*) terhadap fermentabilitas rumput Raja (*P.* hybrid) dengan menggunakan teknik produksi gas *in vitro*. Perlakuan terdiri dari P0 (kontrol/hijauan tanpa imbuhan), P1 (P0+MEY); P2 (P0+MEY+tanin); P3 (P0+Probiotik); P4 (P0+Probiotik+MEY), dan P5 (P0+Probiotik+MEY+tanin) yang disusun dalam rancangan acak lengkap dengan 3 ulangan setiap perlakuan. Suplementasi MEY (P1), probiotik+MEY (P4) dan probiotik+MEY+tanin (P5) nyata (P<0,05) meningkatkan produksi gas hijauan tanpa berpengaruh pada asam lemak volatil (VFA), jumlah protozoa, produksi metana dan kecernaan *in vitro*. Produksi gas tertinggi tercatat pada hijauan yang diberi perlakuan P4 diikuti P5, P1, P2, P3 dan kontrol. Pengaruh perlakuan terhadap kinetika produksi gas menunjukkan perbedaan yang nyata pada inkubasi setelah 8 jam. Walaupun perlakuan hanya berpengaruh terhadap kinetika produksi gas (b, c dan total gas), berdasarkan analisis kluster-hirarki menunjukkan beberapa parameter yang terdiri dari asetat, propionat, kecernaan *in vitro*, jumlah protozoa dan produksi gas metana sangat berkorelasi dengan parameter kinetika produksi gas. Dapat disimpulkan bahwa fermentabilitas hijauan dapat ditingkatkan dengan suplementasi mineral organik maupun kombinasinya dengan probiotik maupun probiotik+tanin tanpa berpengaruh negatif terhadap kecernaan *in vitro*.

Kata Kunci: Fermentabilitas, Kecernaan *In Vitro*, Mineral Organik, Probiotik, Tanin

ABSTRACT

Sofyan A, Sakti AA, Karimy MF, Julendra H, Istiqomah L, Herdian H, Damayanti E, Suryani AE. 2015. Effectivity of probiotic, micromineral enriched yeast and their combination with *Azadirachta indica* leaves containing tannin on fermentability and digestibility of *Pennisetum* hybrid. JITV 20(2): 95-104. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1164

Organic additive for animal had been explored to replace antibiotic growth promoter. Probiotic from lactic acid bacteria was widely used to support the microbial balances in digestive tract, while organic mineral was added into diets to improve bioavailability for preventing mineral deficiency disorders. This experiment was aimed to assess probiotic (*Pediococcus acidilactici* RS2) and micromineral enriched yeast (MEY) combined with tannin from neem (*Azadirachta indica*) leaves containing tannin on king grass (*P.* hybrid) fermentability using *in vitro* gas production technique. Treatments consisted of P0 (control/forage without additive), P1 (P0+MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY), and P5 (P0+Probiotic+MEY+crude tannin). The study was arranged in a completely randomized design (CRD) with three replications in each treatment. Probiotic, MEY or tannin supplementation significantly increased (P<0.05) gas production without affecting volatile fatty acid, protozoa numbers, methane production and *in vitro* digestibility of forage. The highest cumulative gas production was found in forage treated by P4 followed by P5, P1, P5, P2, P3 and control. Kinetic of gas production was significantly affected by treatments after 8 h incubation. Although the treatments were only significantly affected gas production kinetic (*b*, *c* and total gas), the hierarchical cluster analysis indicated that some parameters consisted of acetate, propionate, *in vitro* digestibility, protozoa numbers, and methane production were closely correlated to the gas production kinetic parameters. It was concluded that either organic mineral supplementation or its combination with probiotic, and probiotic+tannin improved fermentabilty of forage without negative effect on *in vitro* digestibility.

Key Words: Fermentability, *In Vitro* Digestibility, Organic Mineral, Probiotic, Tannin

INTRODUCTION

Ruminant contribute to meat and milk supply for human consumption. Low quality feedstuffs is the biggest constrain in ruminant productivity. Supplementation of feed additive such as antibiotic growth promoters (AGP) had been reported could improve the nutrient utilization and animal performance. However, feed additive containing AGP had been banned since January 2006 in accordance with pathogenic resistant risk and antibiotic residues in animal products and by-products (EC 2012).

Feed additive based on organic material had been explored to replace antibiotic in order to support the ruminant performance. Probiotic from lactic acid bacteria are widely used to support the microbial balances in digestive tract. Probiotic is microorganism that supports animal health through maintaining and improving the intestinal microbial balance and its immune response. It is necessary to consider this probiotic, because rumen constitutes an effective animal-microbe interdependency system from that each partner derives benefit to the host animal (Arokiyaraj et al. 2014). The role interdependency system was played by complex microbial community in which the domains bacteria, archaea, and eukarya that responsible for degrading fiber particle from forage as major energy sources (Fraga et al. 2014).

Many species of lactic acid bacteria were used as probiotic cultures for ruminant such as *Lactobacillus animalis*, *L. paracasei*, *Bacillus coagulans* (Agazzi et al. 2014), *Pseudobutyrivibrio ruminis* (Fraga et al. 2014) and *Pediococcus acidilactici* (Cobos et al. 2011; Arokiyaraj et al. 2014). Moreover, Cobos et al. (2011) revealed that *P. acidilactici* isolated from rumen lamb could survive in low pH condition (4.71-4.98) without causing negative effect on dry matter digestibility. It implies that *P. acidilactici* can be used as probiotic.

On the other side, productivity of ruminant was influenced by mineral deficiencies. Although diets was formulated based on the nutrient requirement, in some cases, ruminant metabolic disorder caused by mineral deficiency. Haenlein & Anke (2011) reviewed that mineral deficiency is not only affect productivity parameters but also causes serious disease in ruminant. Organic mineral was added into diets to improve bioavailability for preventing mineral deficiency disorders. Artificial organic mineral (Selenium) involved yeast fermentation had been reported by Gresakova et al. (2013) that Se-yeast had higher absorption in digestive tract and body cell than Se-inorganic form. In addition, Rabiee et al. (2010) reviewed that complex trace mineral in organic form increased ruminant productivity and health.

The other strategy to optimize feed digestibility is through methane reduction by tannin. Neem tree (*Azadirachta indica* A. Juss) is tropical plant, its leaves contain secondary compound such as flavanoid and tannin (Pandey et al. 2014; Bhatta et al. 2015). Many researchers reported that plant secondary metabolites such as tannin could be used to modify ruminal fermentation in which improving feed utilization through reducing methane emission and therefore tannin is considered as a natural compound possessing methane mitigating effect (Bodas et al. 2012; Jayanegara et al. 2012).

Nutrition management strategy for enhancing ruminant production by supplementation of organic feed additive consisting of probiotic and organic mineral was necessary done. The availability of mineral in digestive tract affects the rumen microbe activity. This research was conducted to evaluate addition of probiotic (*Pediococcus acidilactici* RS2) and micromineral enriched yeast (MEY) combined with leaves containing tannin (*A. indica*) on king grass (*P.* hybrid) fermentability using *in vitro* gas production technique.

MATERIALS AND METHODS

Sample preparation

King grass (*Pennisetum* hybrid) was harvested on 70 days after previous cutting (harvest) and used as forage sample, then chopped and dried in oven at 60°C up to reach 12-14% of moisture content. The sample was ground and sieved into two mm particle size. Similarly, this procedure was applied in preparing leaf of Neem (*Azadirachta indica* A. Juss) which was used as tannin source.

Probiotic lactic acid bacteria (*P. acidilactici* RS2) was isolated from cattle rumen and prepared by spray-drying method. Isolate was facultative anaerobically cultivated in deMann Rogosa Sharpe (MRS) Broth media for 18 h at 37°C. The culture was centrifuged at 4500 rpm for 10 minutes, then the pellet/biomass was mixed with the sterilized skim solution (20% w/v) and gum arabic (1% w/v). The solution was homogenized using the digital homogenizer at 8000 rpm for 5 minutes. The culture solution was dried into powder form using the spray dryer (Lab Plant SD-Basic). Spray dryer operating conditions as follows: inlet air temperature was 110°C, outlet air temperature was 55-60°C, and the speed of pump was '3' speed unit. The dried culture viability was evaluated by spread plate method and adjusted to 10^9 cfu/g of bacterial cell density.

Micromineral enriched yeast (MEY) was produced by inoculating *S. cerevisiae* ATCC 9763 into media fortified by micro minerals consisted of Fe, Mn, Cu, Co, Zn, and I. Cassava (*Manihot* sp.) flour as a substrate for fermentation, and the formulation per kg substrate

consisted of $FeCl_2.4H_2O$ (0.177 g), $MnCl_2.4H_2O$ (7.129 g), $CuSO_4.5H_2O$ (9.810 g), $ZnSO_4.7H_2O$ (12.646 g), $CoCl_2.6H_2O$ (0.192 g), and KI (0.217 g). Fermentation was conducted for 7 days in facultative condition then dried in oven at 55°C (up to 24-48 h, DM 10%), followed by ground and sieved into one mm particle size.

Fermentability and *in vitro* digestibility assessment

The sample and rumen liquid were prepared prior to *in vitro* assessment. Two ruminally fistulated Ongole crossbreed cattle adapted by feeding forage (*P*. hybrid) and concentrate (80:20 in dry matter basis) were used as rumen liquor donor. Rumen fluid was taken using aspirator, and immediately transported in pre-warmed vacuum flask (39ºC water temperature) and filtered.

In vitro fermentability was evaluated using *in vitro* gas production technique according to Menke & Steingass (1988). Gas production kinetics was calculated based on the exponential equation according to Ørskov & McDonald (1979). The exponential equation is $P = a+b\ (1-e^{-ct})$ with describing *P* is total gas production, *a* is the gas production from soluble fraction, *b* is the gas production from insoluble fraction, *c* is the rate of gas production, *t* is the time of incubation and *e* is *Euler's* constant (2.7183…). The estimated value of *a*, *b* and *c* were calculated by a fitting curve method using Neway Software developed by Chen (1997). This study was conducted based on Completely Randomized Design (CRD) with six treatments and three replications. Nutrient composition from each treatment was showed in Table 1. The treatments were described as follows:

P0 = control/forage 380 mg (dry matter=92.5%)
P1 = P0+3% micromineral enriched yeast (MEY)
P2 = P0+3% MEY+2% crude tannin
P3 = P0+0.1% probiotic (10^9 cfu/g)
P4 = P0+3% MEY+0.1% probiotic
P5 = P0+3% MEY+ 2% crude tannin+0.1% probiotic

Fermentation was conducted in 100 mL syringe glass (Fortuna model, Poulten and Graft Gmbh Germany). Two syringes containing rumen-buffer without sample (blank) was used in the experiment. All of syringes consisted of samples and blank were randomly incubated for 48 hours in an incubator at 39°C.

Table 1. Nutrient composition of diets consisting of king grass supplemented by either MEY, probiotic or crude tannin

Nutrients	Treatment diets					
	P0	P1	P2	P3	P4	P5
DM	92.5	92.6	92.5	92.5	92.6	92.5
CP (%DM)	11.7	11.4	11.7	11.7	11.4	11.7
CF (%DM)	25.5	25.2	24.8	25.5	25.2	24.8
EE (%DM)	2.1	2.0	2.1	2.1	2.0	2.1
NFE (%DM)	45.0	46.0	46.1	45.0	46.0	46.1
CT (%DM)	-	-	0.28	-	-	0.28

P0 = *P*. hybrid;
P1 = P0+Micromineral Enriched Yeast/MEY;
P2 = P0+MEY+crude tannin;
P3 = P0+Probiotic;
P4 = P0+Probiotic+MEY;
P5 = P0+Probiotic+MEY+crude tannin;
DM = dry matter;
CP = crude protein;
CF = crude fiber;
EE = ether extract;
NFE= nitrogen free extract;
CT = condensed tannin
Nutrient composition was calculated by reference consisted of King grass (Rumiyati 2006); *A. indica* (Obun et al. 2013; Bhatta et al. 2015);
DM content was confirmed by re-analysing in our experiment

Cumulative gas production was recorded at 0, 1, 2, 4, 6, 8, 12, 18, 24, 36 and 48 hours. After 48 h incubation gas was released and the fluid contained in syringe was taken for analysis of protozoa population, VFA, and *in vitro* dry matter and organic matter digestibility. Dry matter (DM) and organic matter (OM) of substrate from each syringe was measured according to AOAC (2012) method. Percentage of DM or OM differences between initial and after incubation and corrected with blank were calculated as *in vitro* digestibility as followed the formula:

$$\text{IVDMD} = \frac{[DMf - (DMr - DMb)]}{DMf} \times 100\ \%$$

and

$$\text{IVOMD} = \frac{[OMf - (OMr - OMb)]}{OMf} \times 100\ \%$$

Where IVDMD: *in vitro* dry matter digestibility, IVOMD: *in vitro* organic matter digestibility, DM*f*: dry matter of feed, DM*r*: dry matter of residue, DM*b*: dry matter of blank, OM*f*: organic matter of feed, OM*r*: organic matter of residue, and OM*b*: organic matter of blank.

Analysis of volatile fatty acid, methane, and counting protozoa

Volatile fatty acid (VFA) product from fermentation was measured according to Sun et al. (2013). Sample was prepared by addition of meta-phosphoric acid and stored at -20°C before analysis. Analysis of VFA was performed by gas chromatography (Shimadzu type 8A) with *packed column* GP10% SP-1200/1% H_3PO4 on 80/100 Chromosorb WAW (Supelco, Bellefonte, PA).

Analysis of methane (CH_4) from gas production was conducted after 18 h of incubation. Ten ml of gas from each sample was taken using vacuum syringe then injected into a vacuum tube. Gas samples were analyzed using gas chromatography (Shimadzu GC-14B) completed with ProparakQ Column (50°C) with helium (He) carrier gas at 60 ml/min flow rate and a flame ionization detector (150°C) as described by Duan et al. (2013). The number of protozoa was counted using hemocytometer and stained with methyl green formalin saline/MFS. MFS solution contained 100 mL of 35% formaldehyde solution, 900 mL of distilled water, 0.6 g of methyl green and 8.0 g of NaCl as described by Sun et al. (2013).

Data analysis

Variables measured were *in vitro* digestibility (IVDMD and IVOMD), fermentability (gas production kinetics, *a*, *b* and *c*), individual volatile fatty acids (VFA), non-glucogenic ratio (NGR), protozoa numbers, methane production (CH_4) and CH4/OMD ratio. The protozoa cell number was converted to logarithmic transformation. Data were evaluated by analysis of variance (ANOVA) and the differences among mean treatments were analyzed using post hoc test of Duncan's Multiple Range Test performed by the CoSTAT statistical software (Cohort 2008).

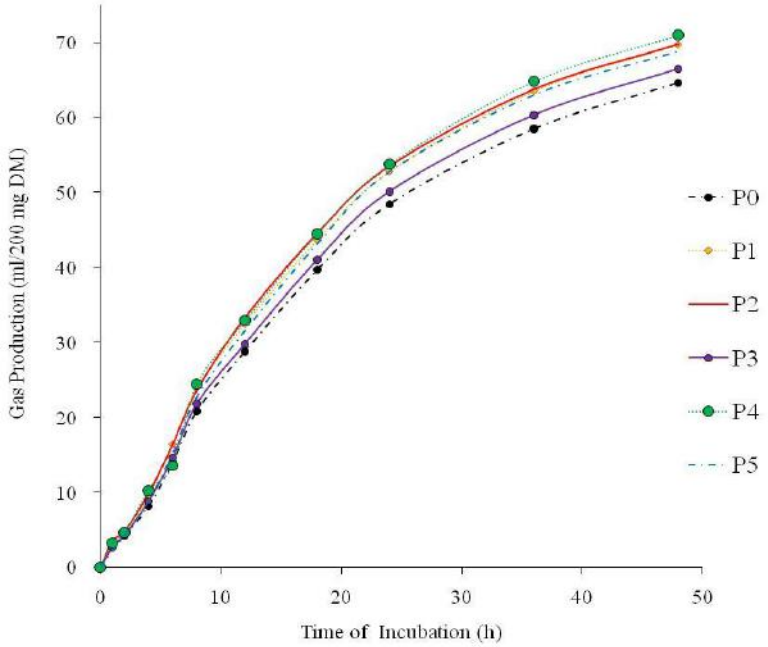
Analysis of interrelationship parameters was performed by hierarchical clustering analysis/HCA (Ametaj et al. 2010). Visualization of HCA was performed using dendogram-heatmap that constructed using `heatmap.2` function from `gplots library` in the R-statistical software (R Core Team 2013). Pre-treatment data were calculated based on the relative differential data from treatments and control using fomula x_t/x_o-1 where $x_t$and x_o denote treatment and control data respectively.

RESULTS AND DISCUSSIONS

Ruminal fermentability characteristics evaluated by *in vitro* gas production were showed on Table 2. Total gas, gas production rate (*c*) and gas production from insoluble fraction (*b*) of forage was significantly affected by treatments ($P<0.05$). The other parameters consisted of gas production from soluble fraction (*a*) or total fraction (*a*+*b*) from forage had tendency ($P\lesssim0.11$) affected by treatments. Forage was supplemented by probiotic+MEY (P4) generated the highest gas production compared to the others.

Gas production generated from the soluble fraction (*a*) resulted negative value that indicated ruminal microbes need adaptation time (*lag* phase) before degrading the insoluble particle. The previous study reported by Arhab et al. (2010) that negative value could be interpreted as *lag* time of ruminal microbes to degrade soluble fraction and then to adhere to cellulosic fraction.

Production of total volatile acid, acetate (C2), propionate (C3), butyrate (C4) and non glucogenic ratio (NGR) indicated no significant difference ($P>0.05$) among treatments (Table 2). Total VFA was varied between 43 ~ 62 mM per mL of rumen fluid. Production of VFA from forage treated by P1, P3, P5, and P4 were 43.2%, 26.2%, 19.3% and 7.7% higher than P2 and control (P0). Non glucogenic ratio seemed constant in all treatments with average value 4.42 except for P5 treatment (13% higher than control), however proportion of C2:C3:C4 was constant about 70:20:10 for all treatments. Based on the ruminal fermentation stoichiometry, individual VFA proportion consisting of C2, C3 and C4 were 60-70%, 20-30% and 10-15% respectively (Wolin et al. 1997).



P0 (*P.* hybrid); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin)

Figure 1. Cumulative gas production of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin incubated during 48 h

In the present study, *in vitro* dry matter (IVDMD), organic matter (IVOMD) digestibility and production of volatile fatty acid (VFA) were not influenced by treatments either probiotic or combination with MEY and tannin. The similar result was also reported by Sun et al. (2013), that probiotic *Bacillus subtilis* supplementation on total mix ration (TMR) did not affect the dry matter digestibility during 24 h, moreover it decreased significantly ($P<0.05$) neutral detergent fiber (NDF) digestibility. In contrast, Fraga et al. (2014) studied that addition of probiotic consisted of *Pseudobutyrivibrio ruminis* significantly increased total VFA concentration of wheat straw. It seems that probiotic supplementation affected *in vitro* digestibility might be influenced by culture adaptation associated with complexity in ruminal ecosystem.

Compared to other animal, supplementation of feed additive complex containing chelated/organic mineral, probiotic, yucca extract indicated that no significant improvement of feed digestibility in horse (Gordon et al. 2013). This varying result could be caused by many factors such as age of animal, viability of probiotic culture, and initial mineral status of the animals.

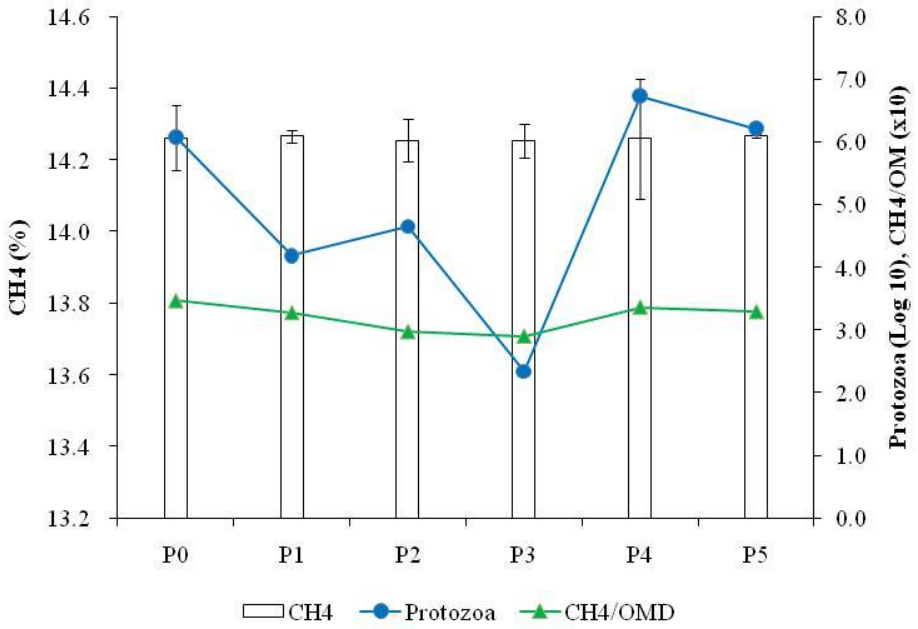
Fermentability evaluated by *in vitro* gas production was indicated by kinetic gas production parameters. Based on the kinetic curve of gas production, all treatments did not significantly influence gas production up to 8 hours of incubation. However, after 8 hours incubation, gas production from forage was significantly affected by treatment (Figure 1). The highest cumulative gas production found at forage treated by P4 followed by P5, P1, P5, P2, P3, and control. It was closely related to the kinetics parameter (*a*, *b*, and *c* values) which indicated that gas production in probiotic treatment and control was lower than others (Table 2).

Methane emission from ruminant reflected the energy lost. The amount of methane production conversely indicated the nutrient utilization. Methane (CH_4) production and methane corrected by organic matter digestibility (CH_4/OMD) during incubation showed similar result in all treatments, around 14.05±0.35% and 32.11±4.25% respectively. CH_4 and CH_4/OMD clearly tended to decrease on treatments P2 and P3.

Table 2. Gas production kinetic, volatile fatty acid production and *in vitro* digestibility of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin

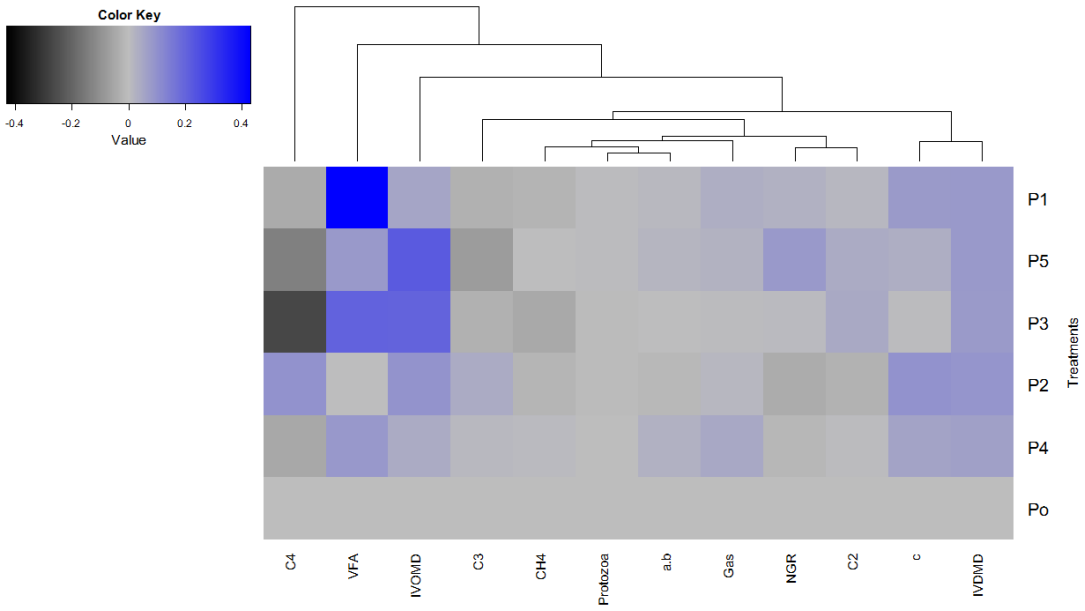
Variables	Treatments						P-value
	P0	P1	P2	P3	P4	P5	
Fermentability/Gas production kinetic parameters							
a (mL)	-0.68±0.27	-0.81±0.14	-0.65±0.29	-0.76±0.15	-0.66±0.10	-1.09±0.06	0.097
b (mL)	47.52±0.61^{ab}	48.12±1.27^{ab}	46.92±0.80^{b}	47.72±0.16^{ab}	48.87±0.48^{a}	48.86±0.67^{a}	0.046*
c (mL/h)	0.042±0.002^{b}	0.046±0.001^{a}	0.047±0.002^{a}	0.043±0.001^{b}	0.045±0.001^{ab}	0.044±0.001^{ab}	0.036*
a+*b* (mL)	46.84±0.35	47.32±1.38	46.28±1.01	46.95±0.08	48.21±0.49	47.76±0.67	0.111
Gas (48h) (mL)	40.25±0.57^{d}	41.71±0.69^{ab}	40.92±0.56^{bcd}	40.39±0.16^{cd}	42.22±0.42^{a}	41.24±0.27^{bc}	0.002*
Volatile fatty acids							
Acetate (mM)	30.55±16.18	43.82±7.36	29.66±11.64	39.28±1.37	33.43±17.34	36.80±10.78	0.684
Propionate (mM)	8.64±4.32	12.10±1.69	8.96±2.82	10.80±0.23	9.12±4.03	9.40±2.93	0.691
Butyrate (mM)	3.97±1.54	5.88±1.27	4.56±1.59	4.39±1.58	3.94±2.04	5.30±2.73	0.587
Total VFA (mM)	43.16±22.03	61.80±10.27	43.19±15.80	54.48±1.39	46.49±23.40	51.50±16.37	0.703
NGR	4.44±0.14	4.58±0.18	4.27±0.28	4.45±0.15	4.38±0.52	5.01±0.16	0.308
In vitro digestibility							
IVDMD (%)	39.40±1.68	42.64±2.82	43.10±3.27	42.58±4.84	41.95±1.02	42.70±1.31	0.638
IVOMD (%)	41.11±1.61	43.46±2.95	45.11±4.33	49.50±12.33	42.80±0.86	50.39±12.09	0.586

P0 (*P*. hybrid); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin). Gas production from soluble fraction (*a*); and insoluble fraction (*b*); rate of gas production (*c*); IVDMD: *In vitro* Dry Matter Digestibility; IVDMD: *In vitro* Organic Matter Digestibility; * Significant Difference



P0 (*P.* hybrid); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+ MEY+crude tannin)

Figure 2. Protozoal cell number, methane production, and methane per organic mater digestibility (CH_4/OMD) of forage supplemented by probiotic, micromineral enriched yeast (MEY) and crude tannin



VFA: volatile fatty acids; C2: acetate; C3: propionate; C4: butyrate; CH4:methane; NGR: non-glucogenic ratio; IVDMD: *in vitro* dry matter digestibility; IVDMD: *in vitro* organic matter digestibility; P0 (*P. hybrid*); P1 (P0+micromineral enriched yeast/MEY); P2 (P0+MEY+crude tannin); P3 (P0+Probiotic); P4 (P0+Probiotic+MEY); P5 (P0+Probiotic+MEY+crude tannin). Color key: (0) denotes no change; (+) denotes value of proportion increase; (-) denotes value of proportion decrease

Figure 3. Dendogram-heatmap visualized hierarchical-clustering analysis of altering ruminal VFA, protozoa, methane production, fermentability, and *in vitro* digestibility

Moreover, protozoa number appeared similar in all treatments was around 6.05 Log10 per mL rumen fluid (Figure 2). Supplementation of probiotic *Propionibacterium* could not alter methane production (Alazzeh et al. 2014). Indeed, Vyas et al. (2014) recommended to combine probiotic supplementation with concentrate to increase molar proportion of propionate and subsequently reduced the CH_4 emissions.

Probiotic from lactic acid bacteria could reduce methane production by the mechanism of formate utilization in rumen, in turn formate would be used by methanogenic bacteria as methane precursor (Jeyanathan et al. 2014). In this study, inability probiotic to reduce methane might be correlated with constraint adaptability of culture in ruminal ecosystem. Similarly, Alazzeh et al. (2014) revealed that probiotic have no effect on methane reduction could be associated with adaptability with other ruminal microbes and including utilization of methane precursor. Moreover, probiotic also did not influence number of protozoa. Both of probiotic and protozoa indicated no antagonistic relationship as similar previously reported by Qadis et al. (2014).

Supplementation of tannin from *A. indica* leaves did not affect the methane production and protozoa number. It might be caused by low dosage of tannin (0.28%) used in the present study. Currently, Bhatta et al. (2015) reported that addition of 2.5% *A. indica* leaves (contained 13.8% of condensed tannin) did not reduced methane production and protozoa number, however the methane production was inhibited when the addition was increase to 25%.

Interrelationship parameters evaluated by hierarchical cluster analysis (HCA) indicated that proportion change of IVOMD, VFA and C4 were higher independently changed by treatment than other parameters (cluster I). Other parameters were categorized into cluster II (C3, CH4, protozoa, *a*+*b*, gas), cluster III (IVDMD and *c*), cluster IV (C2 and NGR). Cumulative gas production and IVDMD had higher similarities related to the change of treatment (Figure 3.). The tree clusters and their shorter *Euclidean* distance indicated higher similarities. Similarity between two metabolites or parameter was represented by branch height (Ametaj et al. 2010).

Organic matter digestibility of forage treated by P5 (probiotic+MEY+tannin) and P3 (probiotic), then total VFA from P1 reflected the higher increased differences than others while forage treated P4 (MEY+tannin) was not affect VFA and gas production. This result revealed that forage treated by probiotic either combining with organic mineral or tannin affected ruminal fermentation. Improving ruminal fermentation by feed additive containing lactic acid bacteria was previously reported Hillal et al. (2011). Otherwise, presence of tannin in P2 (MEY+tannin) resulted in VFA and fermentability lower than P1 might attribute to characteristic of condensed tannin in *A. indica* could inhibit ruminal microbes. Previously, Seresinhe et al. (2012) reported supplementation of condensed tannin could reduce forage fermentability by indicating gas production.

Methane production was closely related to the protozoa number. In the rumen, methane was generated by *Archaea bacteria* that consumed hydrogen. Archea activity was closely symbiosis with protozoa (Bhatta et al. 2015) and endosymbiotic between protozoa and *Archaea* responsible to methane formation (Belanche et al. 2015). Change of NGR was closely related to C2, C3 and C4 in which C2 was dominantly affected due to 60% of VFA. Moreover, gas production and degradation rate of particle affected the IVDMD. Sandoval-Castro et al. (2002) studied the relationship between digestibility and production gas parameter. Significant correlation between gas production and digestibility were influenced by many factors consisted of nutrient composition in which associated microbial ability to adhere and degrade the fraction of feed materials.

Overall, *in vitro* digestibility indicated nutrient utilization in the rumen which was attributed by gas production kinetic parameters and others parameters consisting of VFA, protozoa and methane production. In this study, presence of probiotic complemented by mineral organic or crude tannin had a positive effect on ruminal fermentation of forage.

CONCLUSION

Supplementation of feed additive contained micromineral enriched yeast (MEY) in combination with probiotic *P. acidilactici* RS2 or probiotic+*A. indica* leaves improved fermentability of king grass without affected volatile fatty acid, protozoa numbers, methane production, and *in vitro* digestibility.

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Productivity of *Calliandra calothyrsus*, *Indigofera zollingeriana* and *Gliricidia sepium* on Acid Soil in the Greenhouse

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ABSTRAK

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Tanah masam umumnya kurang baik bagi budidaya tanaman, tidak terkecuali untuk leguminosa pohon karena mengandung Al^{3+} dan Mn^{2+}. Kedua mineral tersebut mungkin bersifat toksik bagi pertumbuhan dan produksi tanaman *Caliandra calothyrsus*, *Indigofera zollingeriana*, dan *Gliricidia sepium* yang umumnya digunakan sebagai hijauan pakan. Penelitian ini bertujuan untuk membandingkan daya toleransi dan produktivitas ketiga jenis tanaman pada tanah masam. Tiga jenis tanaman ditanam di rumah kaca dengan media tanam tanah Ultisol dengan pH 4,57 yang diambil dari perkebunan kelapa sawit Sei-Putih, Medan. Percobaan menggunakan 3 jenis tanaman sebagai perlakuan dan diulang sebanyak 12 kali dengan menggunakan Rancangan Acak Lengkap (RAL). Data dianalisis dengan sidik ragam (ANOVA) menggunakan metode SPSS, program exel, dilanjutkan dengan uji LSD apabila terdapat data yang berbeda nyata. Peubah yang diamati adalah morfologi tanaman, konsentrasi Al^{3+} pada jaringan tanaman, tinggi tanaman, diameter batang, jumlah percabangan pada batang, panjang akar, produksi tanaman, kandungan nutrisi, energi, dan kecernaan *(in-vitro)*. Konsentrasi Al^{3+} pada daun, batang dan akar nyata paling tinggi ditemukan pada *G. sepium*, sedangkan konsentrasi terendah pada *I. zollingeriana. G. sepium* tumbuh lebih kerdil, diameter batang tidak berbeda dengan *C. calothyrsus*, tetapi keduanya lebih rendah dari *I. zollingeriana*. Jumlah percabangan pada *I. zollingeriana*, nyata lebih tinggi dibandingkan dengan *G. sepium*. Panjang akar *C. calothyrsus* tidak berbeda nyata dengan *I. zollingeriana*, sedangkan akar *G. sepium* lebih pendek. Nodulasi akar hanya terbentuk pada *I. zollingeriana.* Produksi biomasa dan kandungan protein tertinggi, serta nilai kecernaan terbaik dicapai pada *I. zollingeriana.* Berdasarkan data kandungan Al^{3+} pada jaringan daun, batang dan akar *I. zollingeriana* nyata lebih toleran terhadap tanah masam *(Ultisol).* Daya toleransi tanaman berpengaruh terhadap pertumbuhan, produksi biomasa, kandungan nutrisi, dan nilai kecernaan yang lebih baik.

Kata Kunci: Avian Influenza, *Cleavage Site*, Hemaglutinin, Patogenitas, Filogenetik

ABSTRACT

Herdiawan I, Sutedi E. 2015. Productivity *of Calliandra calothyrsus, Indigofera zollingeriana* and *Gliricidia sepium* on acid soil in the greenhouse. JITV 20(2): 105-114. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1165

Acid soil which contains Al^{3+} and Mn^{2} is generally unfavorable for crop including the tree legumes. The minerals are toxic to the plants resulted minimalization of growth and crop production. *Caliandra calothyrsus*, *Indigofera zollingeriana*, and *Gliricidia sepium* were tree legumes those are generally used for forage. The aim of this study was to compare their tolerancy to Al^{3+} and growth production on acid soil. The plants were grown in ultisol soil with 4.57 of pH collected from Palm Oil plantation, Sei-Putih, Medan. The experiment was carried out using completely randomized design (CRD) with kind of plants as the treatment and 12 times replication. The data were analyzed by ANOVA using the SPSS and excel program, followed by LSD test when the data was significantly difference. Variables measured were plant morphology, concentration of Al^{3+} in the plant tissues, plant height, stem diameter, number of stem branches, root length, plant production, nutrient content, energy and in vitro digestibility. The highest Al^{3+} contents in leaves, stem and root were significantly observed in those *G. sepium*, while the lowest contents was observed from those of I. zollingeriana. G. sepium was the most dwarf plant and its stem diameter was comparable with the one of *C. calothyrsus*, but was lower than that of *I. zollingeriana*. The highest number of branches was significantly observed in *I. zollingeriana*, while the lowest one was at *G. sepium*. The root length of *C. calothyrsus* was comparable with that of *I. zollingeriana*, while *G. sepium* root was the shorthest one. Root nodulation was only formed at *I. zollingeriana*. The highest biomass production was observed at *I. zollingeriana* which also had highest protein content and the best digestibility. Data from Al^{3+} concentration in tissues of leaves, stems and roots showed that *I. zollingeriana* was the most tolerant plant to acid soils. This tolerancy also affected higher plant growth, biomass production, nutrient concentration, and digestibility.

Key Words: Avian Influenza, Cleavage Site, Hemagglutinin, Pathogenicity, Phylogenetic

INTRODUCTION

Large acid dry soil potential in Indonesia is a chance to produce various crop commodities (food crops, estates, or livestock's feed crops). Several soils that generally had acid pH in the dry area were *Entisols*, *Inceptisols*, *Ultisols*, *Oxisols*, and *Spodosols*, especially for area, which has wet climate with high rainfall. The largest ordos were Ultisol and Inceptisols, with its dominan spreading was in the Sumatera, Kalimantan, and Papua (Mulyani et al. 2004). Utisol was one of soil types which widely spreaded reaching 45794000 ha or about 25% of total of Indonesian land, which was widely used as estate area, among other oil palm, rubber, and industry plantation (Subagyo et al. 2004).

Concentration of high alumunium in the form of Al^{3+} was one of limiting factors of crops cultivation on the acid (pH ≤5.5) land which inhibited crop's growth and production (Gergichevich et al. 2010). In line with it, Sopandie (2006) said that reactive alumunium (Al^{3+}) was released from soil in the form of $AI(OH)^{2+}$ and $AI(OH)^{3+}$ which often become toxic to all agricultural crops, because of the AI ion inhibited root growth quickly in concentration of micromolar. Kinraide & Hagerman (2010) also said that alumunum was very strong toxic to the crop and would inhibit their growth, decrease biomass production and overall crop yield. Ryan & Delhaize (2010) said that Al^{3+} toxicity in acid land (pH ≤5.5) was main factor of stress to the crop, especially to the root tissue of crop that directly contacted with the environtment (Rizonsphere). According to Rengel & Zhang (2003), decreasing of root growth was one of early and very clear simpthom of AI toxication in micromolar (µM) concentration limit which boosted the decreasing of water and nutrient absorption capacity. The alumunium able to inhibit essential nutrient absorption to the crops such as Ca, Mg, Mn, Fe, Mo, and P (Poschenrieder et al. 2008).

Mora et al. (2006) said that AI toxicity changed physiology and biochemistry process of the crop, and its consequence affected its productivity. In despite of Al inhibited process of metabolism and crops growth, but until a certain threshold, tolerant crops (Utama et al. 2005) could tolerate AI effect. According to Wang et al. (2006), several crops were tolerant to aluminum stress because they eliminated AI, so that was not toxic and affected growth and productivity of the crops. Based on Polania et al. (2010), in the genotype of the tolerant crops showed better rooting performance and expected would produce higher biomass. In the context of the sustainable forage on the acid dry land, it needed acid-tolerant foragrs. Several forages included in *Fabaceae* family had good enough tolerance to the dry acid land (Tjelele 2006). *C. calothyrsus*, *I. zollingeriana*, and *G. sepium* were tree leguminous which could be used as forage in the acid soil of estate area, so that needed to be observed extent to which its tolerance and productivity.

MATERIALS AND METHODS

This research was carried out at greenhouse of Agrostology, Ciawi Indonesian Research Institute for Animal Production (AIAT) using 3 tree leguminous (*C. calothyrsus*, *G. sepium*, dan *I. zollingeriana*). Growing media used in this research was Ultiosl acid soil from oil palm plantation, Medan with chemical composition of the soil was presented in the Table 1.

Each of the three crops was planted in plastic pot (40 and 50 cm of diameter, which its base was coated by plastic with 40 cm of diameter to hold water spilled when watering. Planting process was started by seeding of the three crops on the seeding tray for 4 weeks old. After 4 weeks, the seeds were moved into small polybags until 8 weeks old and further, those seedling were moved into plastic pots which were fulfilled by 40 kg of planting media. Watering was done once of 2 days. Volume of watering was adapted with determination result of field capacity (FC). Morphology of crops and root were observed visually at the end of this study. Dry weight production of the crops was done for 44 weeks in every 90 days of harvest day using digital scale. Crops growth was measured in every 2 weeks using meter with 1 cm of scale and digital Vernier calipers. Al^{3+} concentration and nutrient composition was determined from proximate analysis in the nutrition laboratory of IRIAP.

The experiment was carried out using completely randomized design (CRD) with kind of plants as the treatment and 12 times replication. The data were analyzed by ANOVA using the SPSS and excel program, followed by LSD test when the data was significantly difference. Variables measured were plant morphology, concentration of Al^{3+} in the plant tissues, plant height (height, stem diameter, number of branches, root length), plant production (dry weight of leaves, branches and stems, biomass, and ratio of stem/leave) nutrient content (crude protein, crude fiber, fat, dust, Ca, and P), energy and in vitro digestibility of dry and organic materials.

Table 1. Analysis result of soil from oil palm plantation, Sei Putih, Medan

Composition	Soil Samples			
	A	B	C	Average
pH	4.40	4.80	4.50	4.57
Organic material (%)				
Carbon (C)	1.64	1.27	1.56	1.49
Nitrogen (N)	0.16	0.12	0.15	0.14
C/N ratio	10.02	10.06	10.04	10.04
Anion exchange rate				
Ca	7.56	6.99	7.73	7.43
Mg	1.44	2.41	1.32	1.72
K	0.57	0.48	0.43	0.49
Na	0.49	0.51	0.49	0.50
Al^{3+}	1.36	1.15	1.27	1.26

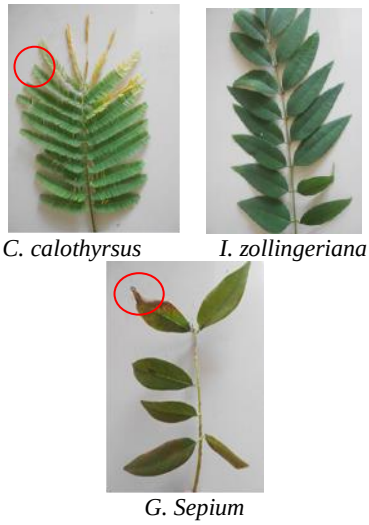
*Analyzed at Soil Laboratory, Indonesian Center for Agricultural Technology Assessment and Development (ICATAD) in 2013

RESULT AND DISCUSSION

Morphology of the crops in the acid soil

Based on observation result, morphologically, *I. zollingeriana* was better than *C. callothyrsus* and *G. sepium*, such as shave and color of leaves, stems, and the number of brunches. Stem of the *C. callothyrsus* seemed thicker than *I. zollingeriana* and *G. sepium*, likewise the leaves color of the I. *zollingeriana* seemed greener than *C. callothyrsus* and *G. sepium* which were yellowish and dry out at the tip of the leaves. *G. sepium* was stunted growth with thin stem, and in almost the leaves had tallow spot, whereas *C. callothyrsus* tree grown spindly and almost all leaves had yellow spot (Figure 1.).

Suntoro et al. (2014) said that the condition of the soil pH is low (acidic), the solubility of some minerals not available to needed for the chlorofil formation.



C. calothyrsus *I. zollingeriana*

G. Sepium

○ = yellow spot

Figure 1. Morfology of leaf in the acid soil

Consequently decreased leaf chlorophyll, leaf color yellow spots which in turn is inhibited the process of photosynthesis rate. Thus the amount of photosynthate produced is very low, this determines the lower plant growth. This showed that both of the *I. zollingeriana* and *C. callothyrsus* were poisoned by micro content. Sumarno (2005) said that clear symptoms of crops that sensitive to the acid soil were very stunted growth, brownish yellow leaves, very limited root growth, minimal flower-shaped, and minimal seed number, very low productivity or failed to produce seeds (Figure 2).

According to Wang et al. (2006) poisoned plant by AI would has nutrient deficiency, such as P, Ca, Mg, Mn, and Fe, so that morphologically was more stunted and its productivity was low. Schaberg et al. (2006) found the same thing in the sugar maple plant that showed high AI content affected low Ca and restricted plant growth. Sumarno (2005) said that the growth of soybean plant on acid soil was suffer due to abiotic and biotic stresses, such as (a) vegetative growth was hampered because of macro and micro deficiency; (b) AI or Mn poisoning; (c) nodule formation was inhibited; (d) the plant was easier to get drought stress; and (e) root growth was inhibited. Furthermore, it was said that very clear symptoms were very stunted growth, brownish yellow leaves, very limited root growth, minimal flower-shaped, and minimal seed number, very low productivity or failed to produce seeds. Although Al concentrations in the nutrient solutions are within the micromolar range (25-1,600 µM), they are sufficient to induce morphological and physiological damage in some crops, and even more significant changes in seedlings (Rengel 1996). Al-toxicity is an important stress factor for plants, limiting plant growth, development and the subsequent performance of commercial crops (Poschenrieder et al. 2008); Rout et al. 2001).

C. calothyrsus

I. zollingeria na

G. sepium

Figure 2. Morphology of the crops in the acid soil

C.calothyrsus

I. zollingeriana

G. sepium

○ = Nodules

Figure 3. Root morphology on the acid soil

Root morphology on the acid soil

Morphology chance of *C. callotyrsus* root was not clearly seen, the roots grown lengthwise, feathers grown normally, but nodule was not found in the main root or the branches. Root morphology of *I. zollingeriana* showed normal growth, the most root hair in every main root or branches and nodule was formed. Root morphology chance was occurred in the *G. sepium*, that was abnormally growth, shorter with slightly root feather and only grown at the root tip (Figure 3).

No formation of nodule on the root of *C. calothyrsus* and *G. sepium* was one indicator of the AL^{3+} poisoning consisted of root cells damage, so that root did not grow well aside from poisoning the environment (rhizosfer) that affected root microbe (rhizobium) growth.

As noted by Taiz & Zeiger (2006) that growth of crops rooting was highly depended on growth environment of the crops and its growth was controlled by crop's activity. Factors affected the soil environment among other factor of physic, biology, and chemistry of soil. The first symptom came up from AI poisoning was short rooting system as a result of cell extension inhibition (Chairani et al. 2007). So that according to Wang et al. (2006) who said that the first response of crop to Al^{3+} poisoning was root tissue damage, so that contributed to nutrient absorption decrease. Besides, AL^{3+} also gave bad effect to structure and function of leaves as photosynthesis machine and showed leaf necrosis, so that assimilation process not running optimally (Zhang et al. 2007).

The highly growth and extension of root under acid soil stress showed higher tolerance than its adaptation to acid soil (nutrient deficiency) with high aluminum content (Polania et al. 2010). Based on Atman (2006), general characteristics of acid soil were pH value of the soil less that 4; low nutrient content of soil organic matter (SOM); low of P availability and Cation Exchange Capacity (CEC) of soil; high content of Mn^{2+} and reactive aluminum (Al^{3+}) that may poison the root and inhibit nodule forming of the legumes. Sudaryono (2009) said that former coal mine land showed pH around 4.4-5.3 was indicated as acid soil, whereas 4.2-4.3 of pH was indicated as very acid soil. The decrease in root growth is one of the initial and most evident symptoms of Altoxicity at micromolar (µM) concentrations in plants (Rengel & Zhang 2003).

AL^{3+} concentration of crops tissue in the acid soil

Average Al^{3+} concentration in tissue of leaves, stems, and roots of *I. zollingeriana* was significantly lowest ($P<0.05$) than *C. callothyrsus* and *G. sepium* (Table 2). The highest Al^{3+} concentration was in the part of root tissue. This was because of the root was a part of crop tissue which directly contacted with rizosphere (acid soil), so that Al^{3+} concentration was accumulated more in the part of that tissue, whereas it was relatively low in the tissue.

Table 2. Al^{3+} concentration of tissue of the three legumes

Legume	Al^{3+} concentration (mg/kg)		
	Leaf	Stem	Root
C. callothyrsus	0.21[b]	26.71[b]	83.65[b]
I. zollingeriana	0.13[c]	14.33[c]	47.77[c]
G. sepium	0.35[a]	52.18[a]	135.51[a]

Not equal letter in the same column shows a significantly difference (P<0.05)

Poisoning symptom was seen from Al^{3+} accumulation in the *G. sepium* tissue, or this crop was not tolerance and disable to eliminate the Al^{3+} accumulation. *I. zollingeriana* and *C. callothyrsus* was able to eliminate the Al^{3+} accumulation on all tissue, so both of the crops still show good morphology character. Delhaize & Ryan (1995) said that crop which tolerance to the Al stress, was a crop which able to accumulate Al fewer, so that Al toxicity was relatively low.

In the soil containing of high aluminum saturation such as several areas in Indonesia, *G. sepium* grew poorly and had low survival. However, Nusantara (2009) said that *Gliricidia* crop was suitable for acid and marginal soils. According to Zang et al. (2007), aluminum in low concentration in soil was very helpful to the growth and would be toxic to the crop only when the concentration exceeds a certain threshold. Furthermore, he said that the highest threshold of the Al concentration was 800 mg/kg in the soil caused decreasing of chlorophyll content of leaves, so that assimilation process was disturbed caused crop productivity decrease. Soil used for this study was 1.26 mol or 34000 mg/kg (Table 1). Ying et al. (2006) reported that low aluminum concentration did not affect or increased the crops growth. On the contrary, Liu et al. (2006), in his study showed that surface area and dry weight of leaves of 2 soybean cultivars increased on the Al concentration treatment as much as 200 mg/kg. Furthermore, on the aluminum concentration of 200-400 mg/kg, the crops started showing assimilation rate decreasing caused by leaf stomata closing. Chen et al. (2006), states that with increasing content of Al^{3+} on the roots and leaves cause the concentration of Mg in the two organs decreases, consequently photosiyntetic active radiation (PAR) was declined.

According to Soemarno (2005), Al concentration in soil solution was very high when soil pH was low. pH value increased on waterlogged soil and Al concentration on soil solution decreased under critical level of Al poisoning. Al stress treatment at Al saturation index of 25% and 50% decreased dry weight of root of 5 soybean genotypes and increased dry weight of Wilis root. The size of the dry weight decreasing of root depended on type of genotype (Hanum et al. 2007).

Crops growth in the acid soil

Result of analysis of variance showed that *C. callothyrsus* was significantly (P<0.05) highest tree (122.47 cm) than *I. zollingeriana* (96.34 cm) and *G. sepium* (62.83 cm) in 44 weeks old (Table 3). Stem diameter of *I. zollingeriana* was significantly (P<0.05) higher by 10.21 mm compared to *C. callothyrsus* and *G. sepium* by 8.99 and 7.54 mm respectively, whereas stem diameter of *C. callothyrsus* and *G. sepium* was not significantly different. Average number of branches of *I. zollingeriana* was significantly (P<0.05) the most by 35.92 branches compared to the other crops, and the lowest was in *G. sepium* by 7.65 branches.

C. callothyrsus root was significantly (P<0.05) longer by 70.36 cm compared to *G. sepium* root by 27.19 cm, but it was not significantly different compared to the *I. zollingeriana*. According to Sumarno (2005), very clear symptoms from the crops which sensitive to acid soil were very stunted growth, tawny leaves, limited rooting growth, flower and seed number forming was minimal, very low productivity or even failed to produce seed. Silveira (2013) said that negative effect of soil acidity to forage growth generally not caused by single factor, but by several factors, which affected normally crops growth. The main factor commonly affected crops growth in the acid soil consisting of toxicity of Hydrogen ion (H^+), aluminum, mangan and essential nutrient deficiency such as phosphor, magnesium, and micronutrient.

Table 3. Growth of the three legumes in the acid soil in 44 weeks old

Legume	*Growth Parameter*			
	High (cm)	Stem diameter (mm)	Average number of branches	Root length (cm)
C. callothyrsus	122.47[a]	8.99[b]	15.88[b]	70.36[a]
I. zollingeriana	96.34[b]	10.21[a]	35.92[a]	69.54[a]
G. sepium	62.83[c]	7.54[b]	7.65[c]	27.19[b]

Not equal letter in the same column shows a significantly difference (P<0.05)

Aluminum was one of soil elements which able to cause poisoning to surrounding plants environment and inhibited the crops growth (Timotiwu 2010). In line with that, Hadiatmi (2002) said that clear symptoms in the shorgum were stunted growth, dwarf, thicker leaves and were dark green with outskirts purplish leaves or dried. Growth of crops rooting very depended on environment and controlled by activity of the crops. Daniel (2011) said that characteristics of aluminum toxicity symptom included of root defects such as thickened, twisted, short root tip and lateral roots, brown root, and did not have a good branching in rooting system.

According to Rout et al. (2001), Al caused disruption of cell fission on root cap and lateral root, cell rigidity through formation of pectin crosslink on the cell wall, and reduced DNA replication through increasing of double chain rigidity. Haling et al. (2011) said that growth and development of big and long crop root under acid land stress showed that capability of tolerance and adaptation to the acidity and saturation of high Al. The first and most recognized effect of Al-toxicity in plants is the inhibition of the division and elongation of meristematic cells and thereby the reduction in root growth (Panda et al. 2003). In line with that, Yoichiro & Midori (2011) said that length root was tolerance indicator of the crops to stress level of aluminum poisoning. Tolerant crops to aluminum would grow well, whereas root of sensitive crops would grow shorter and thick.

Crops production in the acid soil

Dry weight production of *I. zollingeriana* leaves was significantly (P<0.05) higher by 19.23 g/crop compared to *C. callothyrsus* and *G. sepium* by 15.30 and 9.37 g/crop, respectively (Table 4). Dry weight production of *C. callothyrsus* branch/stem was significantly (P<0.05) higher by 13.39 g/crop than G. sepium by 10.20 g/crop, but dry weight production of branch/stem of *I. zollingeriana* and *C. callothyrsus* was not different.

Dry weight production of *I. zollingeriana* biomass was significantly (P<0.05) higher by 32.06 g/crop compared to *C. callothyrsus* and *G. sepium* by 28.70 and 19.58 g/crop, respectively. Leaves/stems ratio of *I. zollingeriana* was significantly (P<0.05) higher by 3.44 compared to *C. callothyrsus* and *G. sepium* by 1.59 and 1.23, respectively. Generally, dry weight production of *I. zollingeriana* was highest than *C. callothyrsus* and *G. sepium*. Chen (2006) and Dewi et al. (2010) said that Al toxicity was the main factor which inhibited crop's productivity in various acid soil throughout the tropics and subtropics. According to Chen et al. (2005b), aluminum stress caused closure of stomata which was responsible to decreasing of CO_2 intake, so that the assimilation rate decrease. It affected decreasing of crop production drastically.

Ma et al. (2002) said that high Al concentration could disturb soybean growth and damage the rooting, so that absorption of nutrient and water was not optimal and caused low productivity of the crop. Based on Hilman et al. (2004), in the acid land, phosphate (P) availability became the major obstacle to increase. Type of the soils was toxic to crops and needed treatments. At pH ≤5.5, Al-toxicity is the main stress factor for plants which limits crop production (Ryan & Delhaize 2010) legume production. Haling et al. (2011), good crop performance under stress of acid soil and drought was caused by capability to tolerate the stress which was implemented in biomass production of canopy and root which was connected with acquisitions level of nutrient and water. Chen et al. (2005a) said that Al decreased CO_2 intake useful in the assimilation process of tangerines (*Citrus rehhni*), which affected to enzyme activity involved in Calvin cycle. The disruption of the assimilation cycle due to the Al induction caused decreasing of nutrition supply to the crop and decreased the production and quality of crop, especially to the sensitive crop. According to Lynch (2013), tolerant crops showed better rooting performance and it was expected would produce higher biomass. Al-toxicity results in alterations of the physiological and biochemical processes of plants and consequently their productivity (Mora et al. 2006).

Nutrition content and digestibility value of the legumes in the acid soil

Crude protein content (Table 5) of *I. zollingeriana* was significantly (P<0.05) highest by 21.80% compared to *C. callothyrsus* and *G. sepium* by 16.80 and 16.64 respectively.

Table 4. Average production per harvest of the three legumes in the acid soil

Legume	Dry weight production (g/crop)			Ratio
	Leaves	Stems	Biomass	Leaves/stems
C. callothyrsus	15.30[b]	13.39[a]	28.70[b]	1.59[b]
I. zollingeriana	19.23[a]	12.83[a]	32.06[a]	3.44[a]
G. sepium	9.37[c]	10.20[b]	19.58[c]	1.23[c]

Not equal letter in the same column shows a significantly difference (P<0.05)

Table 5. Nutrient content and digestibility value of in vitro of the three legumes

Legume	Nutrient content						Gross Energy (Kcal/kg)	Digestibility value (%)	
	CP (%)	CF (%)	CFat (%)	Ash (%)	Ca (%)	P (%)		DMD	OMD
C. callothyrsus	16.80^{b}	30.98^{a}	4.06^{a}	4.18^{b}	0.42^{b}	0.14^{b}	4.472^{a}	59.89^{c}	54.54^{b}
I. zollingeriana	21.80^{a}	23.14^{b}	3.59^{b}	6.62^{a}	1.17^{a}	0.35^{a}	4.184^{b}	73.75^{b}	76.22^{a}
G. sepium	16.64^{b}	23.08^{b}	4.38^{a}	6.08^{a}	0.75^{b}	0.14^{b}	4.162^{b}	78.02^{a}	76.88^{a}

Not equal letter in the same column shows a significantly difference (P<0.05)
CP = Crude Protein
CF = Crude Fiber
CFat = Crude Fat
DMD = Dray Matter Digestibility
OMD = Organic Matter Digestibility

In line with Yayneshet et al. (2009) who said that crude protein content of the forage on the semi-acid land in Ethiopia was decrease drastically caused by stress of drought and soil acidity. Binding of Al^{3+} to cell membrane phospholipids and transport proteins, reduces the net negative membrane surface charge, permitting the movement of anions and restricting that of cations (Huang et al.1992). The highest fiber content was reached by *C. callothyrsus* by 30.98% followed by *I. zollingeriana* and *G. sepium* by 23.14 and 23.08% respectively.

Higher content of structural component (NDF, ADF, and ADL) was found during dry season, especially in the acid soil possibility was caused by lignification height and stadium of crop maturity (Hussain & Durrani 2009).

Ash content of *I. zollingeriana* was significantly (P<0.05) different with *C. callothyrsus* but significantly not different with the *G. sepium*. Similarly, Ca and P content of *I. zollingeriana* was significantly (P<0.05) different with *C. callothyrsus* and *G. sepium*, but Ca and P content of *C. callothyrsus* was significantly not different with *G. sepium*. As said by Zhao et al. (2009) that ash level referred to mineral content closely related to soil condition, soil type, fertilizing and irrigation. Furthermore, Silveira (2013) said that negative effect of soil acidity to forage growth, generally not caused by single factor, but by several factors affected normally crop growth. The main factor generally affected crop growth in the acid soil included hydrogen ion (H^{+}) toxicity, aluminum, mangan, and deficiency of phosphor, magnesium, and micronutrient. Al content could inhibit absorption of essential nutrient, such as Ca, Mg, Mn, Fe, Mo, and P (Poschenrieder et al. 2008). According to Silveira et al. (2011) optimum absorption of the most soil nutrient was occurred when the soil pH was close to neutral. Availability of several macronutrients (N, P, K, S, Ca, and Mg) decreased as an effect of soil acidity increase, so that lime application in the acid soil tended to increase nutrient availability. Al3+ is known to affect cell membrane structure and permeability by blocking the Ca^{2+} channels (Plieth 2005).

Yamamoto et al. (1992) said that inhibition of root growth and development due to Al^{3+} poisoning, in the long term could cause decreasing of capability to absorb the nutrient, suffering from nutrient (P, Ca, Mg, or Fe) deficiency, so that caused bad effect to the growth and development of the canopy. According to White & Broadley (2003), Ca played important role as nutrient in the crops. As a divalent cation, Ca played role as structural wall and cell membrane participated in root and stem growth. Ca deficiency because of Al^{3+} content would affect crop production. Rout et al. (2001) mentioned that Al-induced effects in leaves resemble P deficiencies.

Gross energy value of *C. callothyrsus* was significantly (P<0.05) higher by 4472 kcal/kg than *I. zollingeriana* and *G. sepium* by 4184 and 4162 kcal/kg respectively. According to Dewhurst et al. (2009), gross energy increase of the forage was always in line with dry matter increase, especially to organic matter. Varela de Arruda & Fernandes (2014) said that there was a significant interaction between digestibility of dry material (DM) and gross energy (GE) of the forage which was affected metabolism energy value. Furthermore, it was said by Khachatur (2006) that total content of dry matter of grass that experienced abiotic stress decreased in line with the stress level, as well as its gross energy content.

Digestibility of *G. sepium in vitro* was significantly (P<0.05) highest by 78.02% compared to *I. zollingeriana* and *G. sepium* by 73.75 and 59.89% respectively. Furthermore, digestibility of *in vitro* organic matter of *G. sepium* was significantly not different with *I. zollingeriana* (76.88 vs 76.22), but it was significantly (P<0.05) higher than *C. callothyrsus* (54.54%). Digestibility value of *in vitro* dry matter was the number of dry matter, which could be digested and not excreted in the form of fesses, and it was assumed as absorbed part by the animal (Chuzaemi & Bruchem 1990). According to González & Hanselka (2002),

digestibility value of organic matter of the forage, from wet season to dry season experienced significant decreasing in line with concentration increase of several fiber-forming components. Based on Nisa et al (2004), digestibility value of grass and legume, generally experienced a decreasing by age increase of the plant and soil water content due to concentration increase of crude fiber in the crop tissue, lignification increase, and leaves/stems ratio decrease. Mora et al. (2006) reported that high concentration of Al^{3+} correlated with poor quality of pasture and the higher risk was body weight gain decrease of the animals.

Based on analysis test of nutrient content, all of the legumes planted on the acid soil experienced decreasing from normal condition. Average content of Crude protein of *C. calothyrsus* by 20.0, 23.1, and 25.7% respectively (Tangendjaja et al. 1991; Tangendjaja et al. 1992; Herdiawan et al. (2014). The smallest crude protein decrease was showed by *I. zollingeriana* or become more resistant to acid soil. This may be caused by low cation exchange capacity, so that nutrient absorption experienced small obstacles. Other possibility was a root tissue structural damage caused by Al^{3+} poisoning, so that root absorption effectivity to water and nutrient in the soil was decrease (Khan et al. 2008). Optimum absorption of partly nutrients was occurred when soil pH was close to neutral. Availability of several macronutrients (N, P, K, S, Ca, Mg) decreased as an effect of increasing of soil acidity, so that lime application in the soil acid tented to increase nutrient availability to corn crop (Baligar et al. 1997). It has been reported that Al inhibits the absorption of nutrients, especially Ca, Mg, Fe and Mo and less available P (Poschenrieder et al. 2008).

CONCLUSION

Al^{3+} concentration of *I. zollingeriana* was lower than *C. calothyrsus* or that crop was tolerant to acid soil. Conversely, *G. sepium* was not tolerant causing low growth and productivity. AL^{3+} effect was also seen on root morphology, where nodule forming was only occurred on *I. zollingeriana*. *C. callothyrsus* root was longer with more root hairs resembling *I. zollingeriana*, whereas *G. sepium* root was shorter and the root hair was fewer. *C. callothyrsus* was more tolerant to Al^{3+} than *G. sepium*. Crop height measurement showed that *C. calothyrsus* was highest, but the stem diameter and the number of the highest branches was found on *I. zollingeriana*. The highest biomass was found on *I. zollingeriana*, whereas the fewer biomasses were found on *G. sepium*. Data analysis of nutrient value also showed that *I. zollingeriana* was tolerant to the acid soil and could be developed in that environment.

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Molecular Analysis of Hemagglutinin Gene of Avian Influenza Viruses Isolated in 2012-2013

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ABSTRAK

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Virus Avian Influenza (AI) masih menjadi penyebab utama kasus kematian unggas di Indonesia dan di seluruh dunia. Penelitian ini bertujuan untuk menentukan patogenitas serta melakukan analisis filogenetik dan jarak kekerabatan gen hemaglutinin virus-virus AI yang diisolasi di Indonesia pada tahun 2012–2013 di Jawa Barat, Jawa Tengah, dan Medan. Sampel diperoleh dari peternakan ayam yang mengalami wabah AI dan diinokulasi serta dipropagasi dalam Telur Ayam Berembrio (TAB) Specific Pathogen Free (SPF). Cairan alantois yang dipanen 5 hari setelah inokulasi diuji terhadap aktivitas hemaglutinasi. Cairan alantois dengan hemaglutinasi positif diuji lebih lanjut untuk menentukan subtipe hemaglutinin dan neuraminidase dengan real-time reverse transcription polymerase chain reaction (RRT-PCR) dan dilakukan persiapan untuk sekuensing menggunakan reverse transcription polymerase chain reaction (RT-PCR). Sekuen gen hemaglutinin dianalisis terhadap susunan asam amino di daerah cleavage site dan jarak genetik serta hubungan kekerabatan antar virus. Hasil penelitian mengindikasikan bahwa semua isolat virus tergolong ke dalam HPAI dengan pola asam amino daerah cleavage site QRESRRKKR dan QRERRRKR. Enam isolat termasuk subtipe H5N1 sedangkan 3 isolat lainnya merupakan H5Nx. Semua isolat memiliki hubungan genetik yang dekat dengan jarak genetik kurang dari 0.3 antara virus yang satu dengan yang lainnya dan juga terhadap beberapa isolat virus AI yang menyebabkan wabah-wabah sebelumnya di Indonesia.

Kata Kunci: Avian Influenza, *Cleavage Site*, Hemaglutinin, Patogenitas, Filogenetik

ABSTRACT

Kurniasih SW, Soejoedono RD, Mayasari NLPI. 2015. Molecular analysis of hemaglutinin gene of Avian Influenza viruses isolated in 2012-2013. JITV 20(2): 115-125. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1166

Avian Influenza virus (AIV) still plays as a major cause of the death in poultry in Indonesia and around the world. The aim of this research was to determine the pathogenicity and to analyze the phylogenetic and genetic distances of hemagglutinin gene of isolated AI viruses in Indonesia in 2012-2013 particularly from West Java, Central Java, and North Sumatra. Samples were obtained from poultry farms that suffered from AI outbreaks, were inoculated and propagated in ten days old specific pathogen free (SPF) embryonated chicken eggs. Harvested allantoic fluids at 5 days after inoculation were tested for hemagglutination activity. Positive allantoic fluids were further tested to determine the hemagglutinin and neuraminidase subtype using real-time reverse transcription polymerase chain reaction (RRT-PCR) and to be prepared for sequencing using reverse transcription polymerase chain reaction (RT-PCR). The sequence of hemagglutinin genes were analyzed for the amino acid pattern of the cleavage site region and the genetic distances and relationships of those viruses. The result indicated that all of the isolates are classified as HPAI with the pattern of cleavage site regions are QRESRRKKR and QRERRRKR. Six isolates are classified as H5N1 and 3 isolates are H5Nx. All of the isolates have close genetic relationship with the genetic distances less than 0.3 between one to another and also with several AI viruses that caused previous outbreaks in Indonesia.

Key Words: Avian Influenza, Cleavage Site, Hemagglutinin, Pathogenicity, Phylogenetic

INTRODUCTION

Avian Influenza (AI) disease is potentially very harmful in poultry industry in Indonesia. In spite of the fact that farms had been vaccinated to prevent the outbreak, AI was sporadically still occurred in several areas in Indonesia, event in the poultry that had carried out routine vaccination. Not only in chicken, AI was also reported in ducks and the other waterfowls (Andesfha et al. 2013; OIE 2014).

Indonesian AI viruses are classified in cluster 2.1. Generally, there are 2 clusters of AI virus in the world, that are cluster 1 and cluster 2. Differences in cluster or subcluster may cause in differences of the antigenic structure between one and another virus, therefore vaccine which was used to prevent AI outbreak is

different in one area to another. Phylogenetic analysis is very important to determine the spreads of the virus and genetic distances between AI viruses, so that the prevention and vaccination strategy could be established to prevent the upcoming AI outbreaks (Nidom et al. 2012; Wibawa et al. 2012).

The AI virus belongs to the Orthomyxoviridae family. This virus has 8 gene segments encoding 10 viral proteins. Two types of protein which have important role in pathogenicity of AI virus are hemagglutinin (HA) and neuraminidase (OIE 2014). The HA protein functions for the attachment of the virus to the host cell and to allow fusion between virus membrane and the endosomal membrane of the host cell, whereas neuraminidase has a role in releasing virion progeny into the host cell (Susanti 2008). Hemagglutinin initiates virus infection by binding the receptor of the host cell (Dharmayanti et al. 2012). The hemagglutinin gene contains cleavage site region and other components including antigenic site, receptor binding residue, receptor binding pocket, and glycolisylation site (Susanti 2008).

Cleavage site region is amino acid sequence in hemagglutinin gene where proteases of host cell cleaves the HA0 precursor into the HA1 and HA2 subunits followed by fusion between virus envelope with endosomal membrane of host cell (Perdue 2008). The proteolytic activation of HA molecule is very important in infectivity and virulence of AI virus. The specificity of HA molecule could be the determinant factor to differentiate the pathogenicity of AI virus (Dharmayanti et al. 2012). The differences in HA molecules can be based on amino acid sequence in cleavage site region. Avirulent or low pathogenic AI virus generally has single basic amino acid or arginine (R), whereas virulent or highly pathogenic strains have polybasic amino acid or multiple arginine and lysine (K) (Susanti 2008; Hewajuli & Dharmayanti 2012). This study was aimed to determine the pathogenicity of AI virus isolated in 2012-2013 in several areas in Indonesia and to analyze the phylogenetic and genetic distances between those viruses nor with the isolate of Indonesian AI virus from previous outbreaks.

MATERIALS AND METHODS

Virus isolation

Virus isolates in this study were taken from organs of AI infected chickens (Table 1). Virus isolation was conducted based on the method of Swayne et al. (1998). Twenty grams sample were mashed and added by 80 ml PBS containing Penicillin (10000 IU/ml), Streptomycin (2000 µg/ml), Kanamycin Sulfate (650 µg/ml), and Amphotericin B (20 µg/ml). The suspension was centrifuged for 10 minutes in 5000 rpm. Supernatant obtained was used to inoculate 10 days old specific pathogen free embryonated chicken eggs with 0.2 ml inoculum per egg. The inoculated eggs were incubated in 38-39^{o}C temperature with 60-65% relatives humidity. The incubated eggs were candled to determine the mortality of embryos and dead embryos were stored in 4^{o}C temperature overninght followed by allantoic fluid harvesting. The allantoic fluids were collected into sterile tube and tested for hemagglutination activity, and then stored in -80^{o}C temperature.

Hemagglutination (HA) test

Hemagglutination test was carried out by rapid hemagglutination and microtitration hemagglutination based on Office International des Epizootis (OIE) (2014).

Virus identification by Real-time Reverse Transcription Polymerase Chain Reaction (RRT-PCR)

RNAs of the virus were extracted using QIAamp Viral RNA Mini Kit (Qiagen 2010). Identification of H5 subtype was carried out by RRT-PCR using forward primer 53 5′-ACATGCCCAAGACATACTGGAA-3′, reverse primer H5r-182 5′-GAATTCGTCACACATTGGGTTTC-3′, and probe H5 Probe-79 FAM-CACACAACGGGAAGCTCT-GCGATCT-TAMRA (Chen et al. 2007). PCR composition contained 12.5 µl 2× Quantifast Probe RT-PCR Master Mix, 1 µl primer H5f 0.8 µM, 1 µl primer H5r 0.8 µM, 1 µl probe 0.2 µM, 0.25 µl Quantifast RT Mix, 2 µl template RNA 100 ng, and 7.25 µl Rnase free-water with the total volume 25 µl. PCR process was carried out in the Qiagen Rotor-Gene Q 2plex HRM System with temperature for reverse transcription reaction was 50^{o}C for 10 minutes, initiation/activation of 95^{o}C for 5 minutes, denaturation of 95^{o}C for 15 second, and combination of annealing-extension was 52^{o}C for 60 second with 40 times of the PCR cycle. Identification of N1 subtype was carried out by the same method using forward primer N1F2 5′-GTTTGAGTCTGTTGCTTGGTC-3′, reverse primer N1R1 5′-TGATAGTGTCTGTTATTATGCC-3′, and N1-probe FAM-TTGTATTTCAATACAGCGAC-TAMRA (Payungpon et al. 2006) with annealing/extension temperature was 50^{o}C for 60 second.

Table 1. List of virus isolates 2012-2013

Isolate	Poultry	Origin	Year
A/Ck/Parung Panjang/Prl/2012	Native chicken	Tangerang	2012
A/Ck/Cigudeg/Prl/2013	Native chicken	Bogor	2013
A/Ck/Lyr.Gunungsindur/Prl/2013	Layer	Bogor	2013
A/Ck/Gunungsindur/Prl/2013	Broiler	Bogor	2013
A/Ck/Cianjur/Prl/2013	Broiler	Cianjur	2013
A/Ck/Legok/Prl/2013	Broiler	Tangerang	2013
A/Ck/Medan/Prl/2013	Broiler	Medan	2013
A/Dk/Pakijangan/Prl/2013	Duck	Brebes	2013
A/Dk/Brebes/Prl/2013	Duck	Brebes	2013

Reverse Transcription Polymerase Chain Reaction (PCR) for sequencing

Reverse Transcription-PCR reaction which was used in the sequencing process contained of 10 µl 5× Qiagen OneStep RT-PCR Buffer, 2 µl dNTP Mix 10 µM, 1.5 µl primers 0.6 µM, 2 µl Qiagen OneStep RT-PCR Enzyme Mix, 10 µl Q solution, 2 µl RNA 1-2 µg, and 21 µl RNase-free water with total volume was 50 µl. RT-PCR program consisted of reverse transcription 50^{o}C for 30 minutes, pre-denaturation 95^{o}C for 15 minutes, 40 cycles consisted of denaturation 94^{o}C for 30 second, annealing 53^{o}C for 60 second, and extension 72^{o}C for 60 second, with the final extension 72^{o}C for 10 minutes.

PCR reactions was carried out with 3 different pairs of primer including forward primer HA01 5′-TGGAGAAAATAGTGCTTCTTCTTGC-3′ and reverse primer HA645 5′-GGAAATATAGGTGGTTGGGTTTTG-3′ (Susanti 2008), forward primer HA548F 5′-CCAACCAGAGAAGGATCTTTTGG-3′ and reverse HA1215R 5′-ACTAGGCCTCAAACTGAGTGTTC-3′ (Susanti 2008), primer H5-1 5′-GCCATTCCACAACATACACCC-3′ and H5-3 5′-CTCCCCTGCTCATTGCTA-3′ (WHO 2005), following with the electrophoresis of the PCR products.

Sequencing

Purification of PCR products and sequencing process were done by PT Genetika Science Jakarta and 1st Base Malaysia. The DNA sequences were used for phylogenetic analysis and to determine the pathogenicity of the virus.

Phylogenetic analysis

Phylogenetic and genetic distance analysis among isolates were carried out by multiple alignment ClustalW in BioEdit (Alzohairy 2011). The construction of phylogenetic tree was done by MEGA 5.05 version (Tamura et al. 2011).

RESULT AND DISCUSSION

Death of embryos after AI virus inoculation

Isolate 1, 2, 4, 6, 7, and 8 caused embryo's death in 24-48 hours after the inoculation, whereas isolate 3, 5, and 9 caused embryo's dead after 48 hours (Table 2 and Table 3). The death of embryos was correlated with the virulence and pathogenicity of the virus. Swayne et al. (1998) clarified that pathogenic AI virus can cause embryo's death in 24-48 hours after 0.2 ml inoculation of the virus into allantoic cavity. The death of embryos occurred in 24-48 hours after inoculation in this study indicated that isolate 1, 2, 4, 6, 7, and 8 were pathogenic AI virus. Isolate 3, 5, and 9 caused embryo death after 48 hours. Embryo's death is related to the capability of hemagglutinin gene to be cleaved by the host cell protease. AI virus with pathogenic cleavage site but killed embryos in more than 36 hours was assumed as AI virus which had lost its pathogenicity to its native host. Parallel with the result of Kencana et al. (2014) the death of embryos as the result of non-pathogenic AI virus infection occurred in 3rd day post inoculation. This phenomenon was suspected to be occurred in isolate 3, 5, and 9. The capability of HA protein to be cleaved by host cell protease determines the spreads of

Table 2. Observation of the embryo post AI virus inoculation

<24 hours	24-48 hours	48-72 hours	72-96 hours	>96 hours
live	3 death	-	-	-
live	3 death	-	-	-
live	3 live	2 death, 1 live	1 death	-
live	3 death	-	-	-
live	3 live	2 death, 1 live	1 death	-
live	3 death	-	-	-
live	3 death	-	-	-
live	3 death	-	-	-
live	3 live	3 death	-	-
live	3 live	3 live	3 live	3 live

Table 3. The death of embryo post AI virus inoculation

Isolate	Death of embryo
A/Ck/Parung Panjang/Prl/2012	<48 hours
A/Ck/Cigudeg/Prl/2013	<48 hours
A/Ck/Gunungsindur/Prl/2013	<48 hours
A/Ck/Legok/Prl/2013	<48 hours
A/Ck/Medan/Prl/2013	<48 hours
A/Dk/Pakijangan/Prl/2013	<48 hours
A/Ck/Lyr.Gunungsindur/Prl/2013	>48 hours
A/Ck/Cianjur/Prl/2013	>48 hours
A/Dk/Brebes/Prl/2013	>48 hours

AI virus in chorioallantoic membrane of the embryo. AI virus with HA protein that capable to be cleaved by proteases has the capacity to penetrate the 3 germinal layers of the membranes and enter to the blood vessels (Lebas et al. 2013) and the visceral organs (Krauss et al. 2012). Hemagglutinin from pathogenic AI virus can facilitate the virus spreading from the allantoic space to the deeper mesenchymal vascularization layer of the chorioallantoic membrane, causes the extensive virus infection, contrary with the hemagglutinin from non-pathogenic AI virus that only causes infection in allantoic membrane and inside the allantoic cavity (Feldmann et al. 2000).

Correlation of the virus amount with hemagglutination activity and virulence of the virus

Hemagglutination test is used to determine the quantity of the virus which agglutinates the red blood cell (RBC) and it is a method which is used in the screening of the existence of a causative agent of hemagglutination such as influenza A virus in the isolates harvested from allantoic fluids. The progeny of AI virus which is released from the infected cells can be determined by hemagglutination test (Killian 2014). Agglutination of RBC by AI virus is mediated by reaction between receptor binding site of hemagglutinin molecule with sialic acid receptor of the host cell. Hemagglutinin is a part of virus which will attach on the chicken's RBC receptor causing the agglutination. The attachment would form a protoplasm bridges which eventually form a mass that precipitates in the bottom of the microplate These activities will be the basis in the hemagglutination test to determine the existence of the virus that agglutinates RBC in the allantoic fluids (Natih et al. 2010). HA titre is correlated with the amount of virus in the allantoic fluids after virus inoculation. HA titre shows negative result if the quantity of the virus is less than 10^6 embryo infectious dose (EID_{50})/ml (Kencana et al. 2014). This test is quantitatively, the value of 1 HAU is equal with 10^7 particles of the virus (Killian 2014). Positive reaction of hemagglutination test is occurred if the HA titre is valued ≥2 HAU (Koratkar et al. 2014). HA titre of the isolates in this study (Table 4) were 18-1382 HAU with the highest level was isolate 8 (1382 HAU) and the lowest one was isolate 3 (18 HAU). According to Wanasawaeng et al. (2008), chicken's embryo which was inoculated with virulent AI virus, commonly died within 32 hours with infectivity titre around 7.3-9.0 log2 HA titre or 128-512 HAU. Lang et al. (2011) mentioned that the highest HA titre existed in the allantoic fluids of the embryo harvested at the 1st and 2nd day of the AI virus isolation indicating the increasing of the newly forming infective virion. It could be assumed that isolate 1, 2, 4, 6, 7, and 8 in this study were virulent AI virus with the HA titre ranged 343-1382 HAU.

Table 4. Hemagglutination (HA) titre

Isolate	HA Titre (HAU)
A/Ck/Parung Panjang/Prl/2012	512
A/Ck/Cigudeg/Prl/2013	589
A/Ck/Gunungsindur/Prl/2013	343
A/Ck/Legok/Prl/2013	1024
A/Ck/Medan/Prl/2013	1382
A/Dk/Pakijangan/Prl/2013	512
A/Ck/Lyr.Gunungsindur/Prl/2013	18
A/Ck/Cianjur/Prl/2013	42
A/Dk/Brebes/Prl/2013	74

Moresco (2010) declared that low pathogenic AI virus showed the HA titre less than the amount that could be detected in the allantoic fluids from virus isolation, whereas Koratkar et al. (2014) said that positive reaction of the hemagglutination test occurred if HA titre was ≥2 HAU. Isolate 3, 5, and 9 had positive HA titre or valued as ≥2 HAU, but the HA titre of the isolates were low (around 18-74 HAU). It could be suspected that isolate 3, 5, and 9 were AI viruses which may have experienced a mutation or reasortion that caused the unability to replicate in the host cell to reach the appropriate hemagglutination titre or its hemagglutination activities were lower.

Subtype of avian influenza viruses isolated in 2012-2013

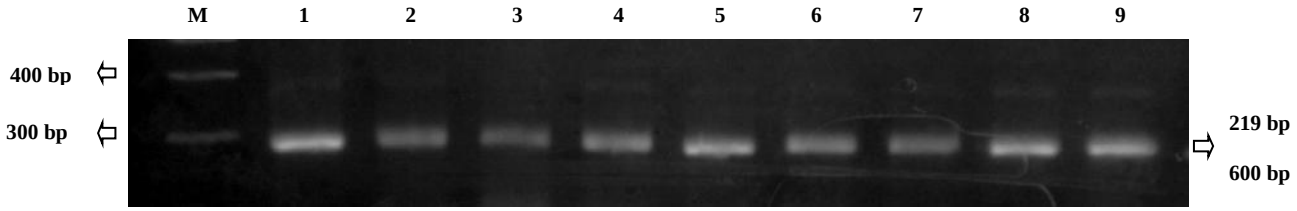
Real Time RT-PCR results threshold cycle (Ct value) which is the amount of PCR cycle at the time when fluorescence increases and can be detected significantly at the early stage of the positive samples. Threshold cycle represents the change of the cycle number at the time when the positive amplification reaction could be measured (Payungporn et al. 2006). The RRT-PCR results positive samples if the Ct value <33, whereas it is negative if Ct value >35 (SADC 2010). In this study, 9 isolates were positive H5. Six from the 9 isolates were positive N1, therefore the six isolates (isolate 1, 2, 4, 6, 7, and 8) could be classified into H5N1 subtype, whereas the other 3 isolates (isolate 3, 5, and 9) were H5 subtype aside of N1 (H5Nx subtype). The results of the RRT-PCR test were presented in the Table 5.

The results of RRT-PCR in this study showed 10^6-10^8 copies of RNA of the H5 positive isolates and 10^5-10^8 copies of RNA of the N1 positive isolates. RRT-PCR product was detected using specific sequence probe to amplify only the specific target. Specific HA probe could be used for the quantification of the virus with different subtypes in one virus mixture (OIE 2014; Spackman 2014). The amount of RNA copy that could not be read at the N1 RRT-PCR test from isolate 3, 5, and 9 indicated that there were no amplification occured, and the isolates were considered to be negative N1. However, Ct value and the amount of RNA copy did not show correlation with the result of virus isolation and hemagglutination test. The H5Nx isolates apparently had Ct value and RNA copy comparable with isolates from H5N1 subtype. This was possible because RRT-PCR test has the high sensitivity and specificity that can detect and amplify the very small quantity of RNA. The difference in AI virus detection between RRT-PCCR and virus isolation is caused by the difference of the ability of both test to detect the parts of the virus. Virus isolation only detects live virus and cannot detect virus that had been inactivated or had experienced other treatments, whereas the RRT-PCR can detect live or inactivated virus (Spackman 2014)

Table 5. Threshold cycle (Ct value) and number of RNA copy of H5 and N1 in RRT-PCR

Isolate	Ct		RNA copy /µl	
	H5	N1	H5	N1
A/Ck/P.Panjang/Prl/2012	20.02	13.90	1381543	283112136
A/Ck/Cigudeg/Prl/2013	17.22	15.16	63815531	125437247
A/Ck/Gunungsindur/Prl/2013	16.54	23.75	92686366	481532
A/Ck/Legok/Prl/2013	15.94	16.12	128404046	67493217
A/Ck/Medan/Prl/2013	16.18	15.14	112597534	126602979
A/Dk/Pakijangan/Prl/2013	17.06	17.81	6975631	22448560
A/Ck/Lyr.Gnsindur/Prl/2013	16.89	tba	76147115	tba
A/Ck/Cianjur/Prl/2013	17.85	tba	45025043	tba
A/Dk/Brebes/Prl/2013	20.54	tba	10331331	tba

[a]Result could not be read by the thermalcycler



M. Marker 1kb; 1. A/Ck/Parung Panjang/Prl/2012; 2. A/Ck/Cigudeg/Prl/2013; 3. A/Ck/Lyr.Gunungsindur/Prl/2013; 4. A/Ck/Gunungsindur/Prl/2013; 5. A/Ck/Cianjur/Prl/2013; 6. A/Ck/Legok/Prl/2013; 7. A/Ck/Medan/Prl/2013; 8. A/Dk/Pakijangan/Prl/2013; 9. A/Dk/Brebes/Prl/2013

Figure 1. PCR product of H5-1 H5-3 primer amplification

In the other side, in connection with the mortality of the embryo and HA titre, the results of the RRT-PCR test had a correlation with virus isolation and hemagglutination test. RRT-PCR test, technically accurately measured RNA quantity correlated with HA content (Spackman & Suarez 2005). Isolate 3, 5, and 9 showed embryo death after 48 hours and had lower HA titre (18, 42, and 74 HAU respectively) were H5Nx subtype, therefore could be assumed that only isolates classified in H5N1 subtype that could kill embryo in 24-48 hours and had higher HA titre (343-1382 HAU) compared to H5Nx subtype.

Amino acid sequences of the Cleavage site of hemagglutinin gene

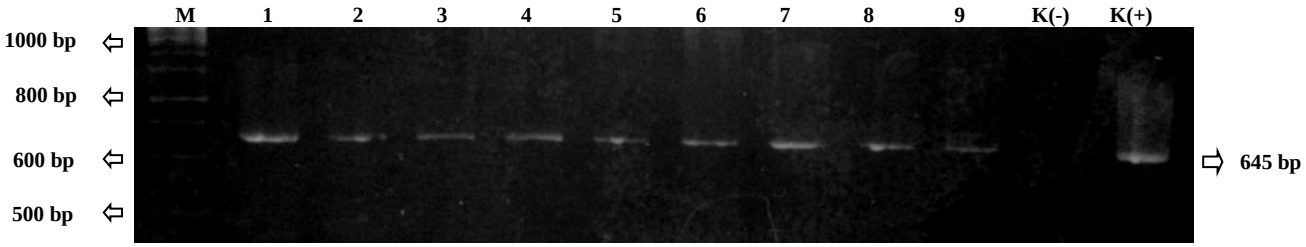
The electrophoresis of the PCR product is presented in Figure 1. Sequencing result of the PCR products with primer that specificaly amplified cleavage site region of hemagglutinin gene showed sequence of repeated arginine (R) and lysine (K) amino acid (Table 6). AI virus with polybasic amino acid sequence of arginin or lysine are classified as pathogenic (Gohrbandt et al. 2011; Li et al. 2011). Virulence characteristic of AI that distinguish HPAI from LPAI is the ability of the virus to be cleaved by proteases which could be found in almost all type of host cells. Hemagglutinin gene produced as single polypeptide is cleaved into HA1 and HA2 subunit before the virus become infectious. This cleavage process is important for the fusion domain to be active during virus replication process and facilitate AI virus infection to the host cell. Generally, cleavage process is conducted by trypsin or trypsin-like proteases which cleaved HA protein due to the recognition of single arginine. Monobasic (single arginine) amino acid sequence could be cleaved by tryptase produced by ephitelial cell of gastrointestinal and respiratory tracts, therefore the AI virus infection is restricted in gastrointestinal and respiratory organs. However, if polybasic amino acid or repeated arginine or lysine are existed, the cleavage site becomes accessible to furin or other ubiquitous proteases, such as proprotein convertase 6 (PC6) in the Golgy that is found in most

Table 6. Amino acid sequence of hemagglutinin gene on cleavage site

Isolate	Amino acid pattern
A/Ck/Parung Panjang/Prl/2012	QRESRRKKRG
A/Ck/Cigudeg/Prl/2013	QRESRRKKRG
A/Ck/Gunungsindur/Prl/2013	QRESRRKKRG
A/Ck/Legok/Prl/2013	QRESRRKKRG
A/Ck/Medan/Prl/2013	QRESRRKKRG
A/Dk/Pakijangan/Prl/2013	QRESRRKKRG
A/Ck/Lyr.Gunungsindur/Prl/2013	QRERRRKRG
A/Ck/Cianjur/Prl/2013	QRERRRKRG
A/Dk/Brebes/Prl/2013	QRERRRKRG

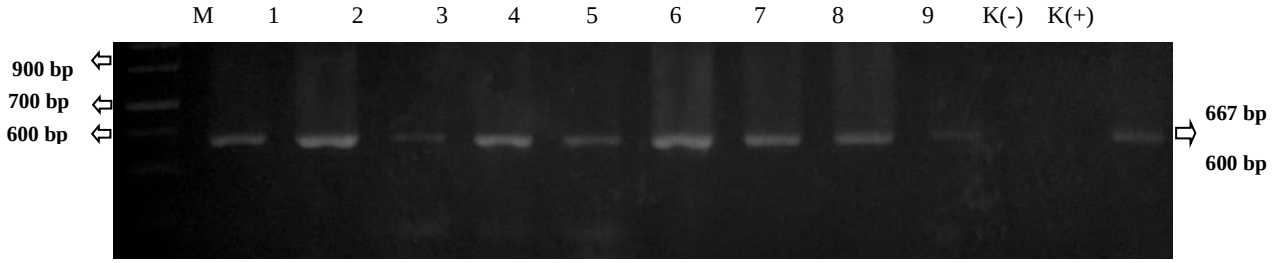
cells and so the infection could be occurred in the various tissues and infect systemically. Therefore, amino acid sequence in the cleavage site determines the pathogenicity of AI virus, as AI virus could be classified into highly pathogenic (HPAI) or low pathogenic (LPAI) (Bogs et al. 2010; Gohrbandt et al. 2011). The cleavage site region of hemagglutinin gene plays very important role in producing infectious viral progeny during the AI virus infection (Leijon et al. 2011). Polybasic amino acid or arginine and lysine in the hemagglutinin of H5N1 AI have a role in systemic infection, so that AI virus could be isolated from blood, brain, nerve, cerebrospinal fluid, cornea, heart, lungs, kidney, pancreas, intestine, caecum, and feces (Yamamato et al. 2010; Kim et al. 2015).

The research of Wibawa et al. (2011) in ducks in Central Java in 2007-2008 showed amino acid patterns of cleavage site were QRERRRKKR, QRESRRKRR, QRESRRRKR, QKESRRKKR, and QRESRRKKR. Wibawa et al. (2012) also isolated AI virus that caused outbreak in duck in several areas in Central Java, Yogyakarta, and East Java in September-November 2012 and showed amino acid patterns of the cleavage site was QRERRRKR. Research of Andhesfa et al. (2013) of birds in Central Java, Yogyakarta, and East



M. Marker 1kb; 1. A/Ck/Parung Panjang/Prl/2012; 2.A/Ck/Cigudeg/Prl/2013; 3. A/Ck/Lyr.Gunungsindur/Prl/2013; 4. A/Ck/Gunungsindur/Prl/2013; 5. A/Ck/Cianjur/Prl/2013; 6. A/Ck/Legok/Prl/2013; 7. A/Ck/Medan/Prl/2013; 8. A/Dk/Pakijangan/Prl/2013; 9. A/Dk/Brebes/Prl/2013; K(-). Negative control, K(+). Positive control

Figure 2. PCR product of HA01-HA645 primer amplification



M. Marker 1kb, 1. A/Ck/Parung Panjang/Prl/2012; 2.A/Ck/Cigudeg/Prl/2013; 3. A/Ck/Lyr.Gunungsindur/Prl/2013; 4. A/Ck/Gunungsindur/Prl/2013; 5. A/Ck/Cianjur/Prl/2013; 6. A/Ck/Legok/Prl/2013; 7. A/Ck/Medan/Prl/2013; 8. A/Dk/Pakijangan/Prl/2013; 9. A/Dk/Brebes/Prl/2013; K(-). Negative control, K(+). Positive control

Figure 3. PCR product of H548F-H1215R primer amplification

Java in 2011-2012 also explained that AI virus which successfully isolated had amino acid pattern of the cleavage site of QRESRRKKR and QRERRRKR and caused AI outbreaks in those areas.

Sequencing analysis result of the hemagglutinin cleavage site of the AI viruses in this study showed that 6 isolates (H5N1) had amino acid pattern of QRESRRKKR and 3 isolates (H5Nx) had QRERRRKR. QRESRRKKR pattern by previous scientist was reported to be found in waterfowls, whereas in this study the pattern was found in AI isolate which cause outbreaks in broiler chicken (A/Ck/Gunungsindur/Prl/2013, A/Ck/Legok/Prl/2013, A/Ck/Medan/Prl/2013), native chicken (/Ck/Parung Panjang/Prl/2012, A/Ck/Cigudeg/Prl/2013), and duck (A/Dk/Pakijangan/Prl/2013). It was assumed that AI virus that caused the outbreaks were came from ducks or other waterfowls wandering around the farm or kept in backyard which clinically was healthy and became as a source of transmission of AI virus. WHO (2011) and Coker et al. (2014) explained that wild or domestic waterfowls has important role in AI virus transmission to the other domestic birds. Wild waterfowls is a natural host of the type A influenza virus and plays important role in the ecology and virus propagation. Commonly from the natural host, the type A influenza virus could be transmitted to the other birds (Pillai et al. 2010). AI virus is replicated in epithelial cells of the intestinal track of the wild waterfowls. Virus excretion through feces could become the source of transmission of AI virus to other domestic birds. Duck's living place which is close to the water environment makes it possible to be a media of virus transmission through the water (Hewajuli & Dharmayanti 2012). When AI virus has been transmitted by duck to the other birds, there would be a high morbidity, clinical symptoms, and mortality rate (Henning et al. 2010; Leijon et al. 2011). This also showed that waterfowls which clinically healthy play as an evolution place of AI virus and by time the virus become more pathogen (Henning et al. 2010; Hewajuli & Dharmayanti 2012).

The QRERRRKR amino acid sequence of 3 isolates of H5Nx resembled with the common pattern existed in duck, is in accoordance with Wibawa et al. (2012). In this research, the same pattern was found in commercial layer (A/Ck/Lyr.Gunungsindur/Prl/2013), commercial broiler (A/Ck/Cianjur/Prl/2013), and duck (A/Dk/Brebes/Prl/2013). It was assumed that the waterfowls also played as a source of transmission in those cases. However, those 3 isolates had phenotypic difference with another 6 isolates in case of the longer period was needed to cause embryo death and the lower HA titre. There was a possibility that the viruses were came from low pathogenic virus which had undergone mutation or reasortion, so that had amino acid pattern in the cleavage site of hemagglutinin gene resembled with the pattern of the pathogenic virus. Gohrnandt et al. (2011) explained that a HPAI strain may be an

acquisition of a non-pathogenic AI strain which had experienced a mutation and had cleavage site region that characterized pathogenic AI strain. In spite of it did not show clinical symptoms, the waterfowls could continuously excrete virus, so it could be potentially mode of spreading pathogenic virus to another birds (Li et al. 2011). Zhao et al. (2012) said despite an AI virus having cleavage site with the amino acid pattern characterized of high patogenicity AI virus, however phenotypically the virus could be characterized as low pathogenicity.

The fact that 3 isolates of H5Nx had phenotypic resembling low pathogenic AI virus but evidently having amino acid sequence of QRERRRKR which was characterized as high pathogenic virus, confirmed the assumption that AI viruses that had been isolated in Indonesia were pathogenic viruses, in line with WHO (2011) and Wibawa et al. (2012) which reported that AI viruses circulated in Indonesia since first report in 2003 were highly pathogenic avian influenza (HPAI) H5N1 subtype and that the prevalence of low pathogenic AI virus in Indonesian poultry had not been found yet.

Phylogenetic Analysis and Genetic Distance

The electrophoresis of the PCR product is shown in Figure 2 and Figure 3. Phylogenetic analysis and genetic distances which described in Figure 4 and Table 7 showed that AI isolates in this study were divided into 2 clusters. Isolate 1, 2, 4, 6, and 7 were in same cluster with AI isolated from chicken in Banten in 2008 (GenBank: GU183461), Legok 2003 (GenBank: GU052426.1), and AI Legok isolated by Prolab in 2008. The Legok 2003 was the isolate obtained from the first AI outbreak in Indonesia, so it was assumed that isolate 1, 2, 4, 6, and 7 were the descendant of the AI virus that caused the first outbreak and had not a meaningful genetic mutation. Genetic distance between the isolates ranged 0.042-0.081. Isolate 8 and 9 which were isolated from duck and isolate 3 and 5 which were isolated from layer and broiler chicken had genetic similarity with duck isolated from Tegal 2012 (GenBank: KC417274.1) and Blitar 2012 (GenBank: KC417277.1). Genetic distance between the isolates was 0.003-0.029. The genetic similarity between AI viruses isolated from chicken with those were isolated from waterfowls was suspected due to of the existence of waterfowls reared around the chicken farms resulting in AI transmission from the waterfowls to the chicken. Henning et al. (2010) said that waterfowls is the source of transmission of AI virus to another surrounding birds. Water as a living place of the waterfowls become the media and source of AI virus infection. The fact that waterfowls are the main source of AI virus infection makes the implementation of prevention and control programs of AI virus become more difficult to be conducted (Hewajuli & Dharmayanti 2012). The genetic distance between the 2 clusters in this study was 0.062-0.131, this showed that all of the isolates in the 2 clusters still had close relationship with the coefficient of phylogeny was <0.3 and homology was >97% (Wibawa et al. 2012).

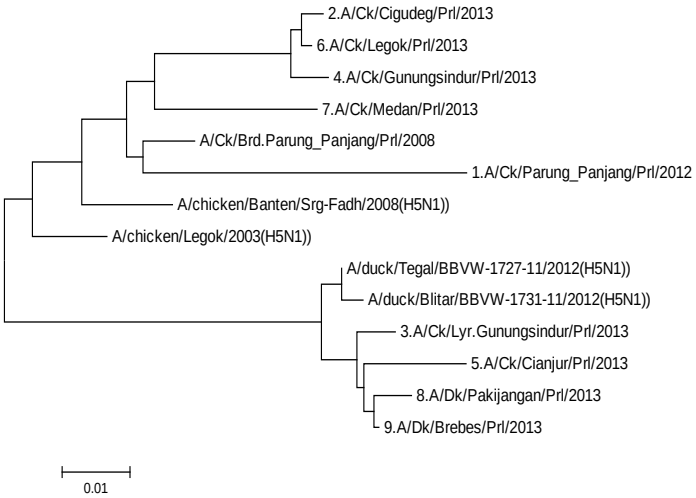


Figure 4. Phylogenetic tree of the hemagglutinin gene of AI viruses isolate 1-9 compare with AI virus from the previous outbreaks

Table 7. Genetic distances of the hemagglutinin gene between AI viruses used in the research with AI viruses from previous outbreaks

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
[1]															
[2]	0.025														
[3]	0.071	0.062													
[4]	0.074	0.065	0.003												
[5]	0.029	0.042	0.087	0.090											
[6]	0.066	0.080	0.129	0.131	0.055										
[7]	0.055	0.062	0.103	0.106	0.045	0.081									
[8]	0.085	0.076	0.013	0.016	0.080	0.125	0.103								
[9]	0.053	0.060	0.101	0.104	0.048	0.081	0.011	0.099							
[10]	0.096	0.083	0.025	0.029	0.086	0.127	0.106	0.022	0.108						
[11]	0.055	0.062	0.103	0.106	0.042	0.076	0.005	0.099	0.008	0.100					
[12]	0.049	0.059	0.107	0.110	0.038	0.071	0.043	0.103	0.053	0.108	0.045				
[13]	0.089	0.076	0.016	0.019	0.082	0.124	0.097	0.014	0.101	0.022	0.097	0.097			
[14]	0.085	0.073	0.013	0.016	0.078	0.120	0.093	0.008	0.095	0.017	0.093	0.097	0.006		

[1] #A/chicken/Banten/Srg-Fadh/2008(H5N1)); [2] #A/chicken/Legok/2003(H5N1)); [3] #A/duck/Tegal/BBVW-1727-11/2012(H5N1)); [4] #A/duck/Blitar/BBVW-1731-11/2012(H5N1)); [5] #A/Ck/Brd.Parung_Panjang/Prl/2008; [6] #1.A/Ck/Parung_Panjang/Prl/2012; [7] #2.A/Ck/Cigudeg/Prl/2013; [8]#3.A/Ck/Lyr.Gunungsindur/Prl/2013; [9] #4.A/Ck/Gunungsindur/Prl/2013; [10] #5.A/Ck/Cianjur/Prl/2013; [11] #6.A/Ck/Legok/Prl/2013; [12] #7.A/Ck/Medan/Prl/2013; [13] #8.A/Dk/Pakijangan/Prl/2013; [14] #9.A/Dk/Brebes/Prl/2013

CONCLUSION

All of the isolates used in this study were highly pathogenic avian influenza (HPAI). Six isolates were classified into H5N1 subtype, whereas the other 3 isolates were included into H5Nx subtype. Phylogenetic analysis and genetic distance between viruses showed that isolates in this study were divided into 2 clusters and still had close relationship.

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The Reliability of DIVA Test Based on M2e Peptide Exceed Those Based on HA2 or NS1 Peptides

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ABSTRAK

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Salah satu kelemahan utama vaksinasi pada avian influenza adalah bahwa vaksinasi tersebut tidak dapat memberikan perlindungan terhadap infeksi. Bila peternakan yang menerapkan vaksinasi terpapar virus dalam jumlah besar, infeksi subklinis dapat menyebar dalam peternakan tersebut dalam waktu yang lama tanpa diketahui. Kondisi tersebut hanya dapat dimonitor dengan alat tes DIVA (*differentiation of infected from vaccinated animals*), alat uji konvensional tidak dapat digunakan. Tes DIVA berdasarkan antibodi yang terbentuk akibat stimulasi virus bereplikasi merupakan tes DIVA yang paling sesuai. Untuk influenza H5N1 antibodi yang dimaksud antara lain antibodi terhadap M2e, protein NS1 dan peptida HA2 (HA_488-516). Tujuan penelitian ini adalah membandingkan level antibodi terhadap peptida M2e, NS1 dan HA2 pada ayam normal, vaksinasi dan infeksi (1, 2-3, ≥4 minggu pasca infeksi). Level antibodi diukur dengan ELISA menggunakan sintetik peptida sebagai antigen koting. Peptida yang digunakan antara lain: 4 buah peptida NS1 yang didasarkan pada berbagai lokasi pada protein NS1, peptida M2e dan HA2. Semua peptida dibiotinilasi pada ujung N nya. Koting peptida pada microtitre plate dilakukan secara langsung atau melalui jembatan streptavidin. Penelitian ini menunjukkan bahwa vaksinasi tidak merangsang pembentukan antibodi terhadap semua peptida. Ayam yang terinfeksi membentuk antibodi dengan level yang tinggi terhadap peptida M2e, tetapi sangat rendah terhadap peptida NS1 dan HA2. Antibodi terhadap peptida NS1 dan HA2 hanya dapat dideteksi dengan ELISA streptavidin-peptida. ELISA berbasis NS1 atau HA2 tidak dapat diandalkan sebagai tes DIVA untuk penyakit AI H5N1 pada ayam.

Kata Kunci: M2e, Protein NS1, Peptida HA2, Tes DIVA, H5N1

ABSTRACT

Tarigan S, Sumarningsih, Ignjatovic J. 2015. The reliability of DIVA test based on M2e peptide exceed those based on HA2 or NS1 peptides. JITV 20(2): 126-133. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1167

One of the most important disadvantage of vaccination against avian influenza is that it cannot protect vaccinated birds against infection. When vaccinated poultry are heavily exposed to the virus, prolonged, unrecognised, subclinical infection may persist on the farm. The condition can only be serologically monitored by a DIVA (differentiation of infected from vaccinated animals) test, whereas conventional diagnostic tests cannot be used. The DIVA tests based on an antibody response following virus replication is the most appropriate approach. For H5N1 influenza such antibodies includes those to the M2e and NS1 proteins and an epitope on the HA2 subunit (HA_488-516). The purpose of this study was to compare the magnitude of the antibody response in chickens vaccinated and infected with an H5N1 virus strain. For that purpose, sera collected from naïve, vaccinated and infected birds, at 1, 2-3, ≥4 weeks post challenge were used. Antibodies were measured by ELISA using biotinylated synthetic peptides as coating antigens. The peptides used include four NS1 peptides corresponding to different regions of the NS1 protein and HA_488-516and M2e peptides. Peptides were coated onto microtitre plates either directly or via a streptavidin bridge. The results showed that vaccination did not cause antibody conversion to any of the peptides, where as challenged birds developed a high antibody response to M2e but, low response to the NS1 and HA2 peptides. Antibodies to the later peptides were detected only by the streptavidin-peptide ELISA. The ELISA based on NS1 or HA_488-516 peptides, therefore, are not reliable for use as DIVA test in H5N1 avian influenza virus infection.

Key Words: M2e, NS1 Protein, HA2 Peptide, DIVA Test, H5N1

INTRODUCTION

One of the most important draw backs of avian influenza vaccination in poultry is the difficulty in disease surveillance. Vaccination provides protection against clinical disease and reduces the amount of virus shed by the infected birds, but it cannot provide complete protection against virus infection (Savill et al.

2006). When a vaccinated flock is exposed to HPAI virus challenge, it may result in subclinical infection that continues to circulate in the flock unrecognized by the farmers. This situation is compounded by the fact that a practical diagnostic tool is not available that can be used to detect the presence of subclinical infection. The common serological test such as HI test, ELISA or Agar gel precipitation test could not be used, as they are unable to differentiate between antibodies in birds due to infection and those due to vaccination. Virus isolation, PCR or rapid antigen detection test are either too expensive or insensitive.

Serological test that can identify infected among vaccinated birds are termed DIVA (Differentiation of Infected from Vaccinated Animals) test. Different DIVA strategies for avian influenza have been reviewed previously (Suarez 2005; Tarigan 2015). DIVA strategy based on heterologous-neuraminidase was successfully applied in Italy (Capua et al. 2003; Capua et al. 2004). The prerequisite was that the vaccine used had to be generated from virus subtypes with neuraminidase heterologous to that of circulating virus, preventing the application of this strategy in other countries, including Indonesia. In addition, the indirect iFAT (fluorescent antibody test) used to identify infected birds in this strategy is also not practical.

Unlike DIVA test based on heterologous-neuraminidase, DIVA test based on the external domain of M2 protein (M2e) is independent from the vaccine subtype since antibodies to M2e develop only in infected animals but not in vaccinated with killed AIV vaccines. ELISA based on either synthetic M2e peptide or bacterial expressed M2e protein has been used to measure the M2e antibody response (Lambrecht et al. 2007; Kim et al. 2010; Hemmatzadeh et al. 2013; Hadifar et al. 2014; Tarigan et al. 2015). The M2e-ELISA was reported to have high specificity but rather low sensitivity. The low sensitivity is attributed to the fact that M2e antibody can be detected in birds not sooner than 7 days of infection, and also some birds fail to seroconvert to M2e. Additional DIVA test capable of detecting infection in those M2e-negative, but infected birds, would be advantageous in order to increase the sensitivity of DIVA testing. The purpose of this study was to investigate the possibility of an ELISA test based on the influenza virus nonstructural-1 (NS1) protein and also on the recently identified epitope in the HA2 domain of haemagglutinin H5 (HA_488-516) as possible supplements to the M2e DIVA test (Khurana et al. 2011). This possibility was investigated by comparing the magnitude of the antibodies to those proteins or peptides in naïve, vaccinated and infected chickens.

The NS1 protein is the only true non-structural protein in Influenza viruses and interest in developing DIVA tests based on this protein have been considered. However, the accuracy of test based on this protein as DIVA test is still contradictory between different studies. Some studies found that the NS1 based tests are sensitive and specific as DIVA test (Birch-Machin et al. 1997; Golchinfar et al. 2014; Ozaki et al. 2001; Takeyama et al. 2011), while others have found the reverse (Avellaneda et al. 2010). In this study, the accuracy of NS1-based ELISA as DIVA test was evaluated thoroughly by analyzing different fragments of the protein and different condition of ELISA. In addition, antibody to an epitope in the HA2 domain of haemagglutinin H5 (HA_488-516) suggested as being a reliable marker for H5N1 infection in human, has yet to confirmed in poultry (Khurana et al. 2011).

MATERIALS AND METHODS

Peptide

Four NS1 peptides, one HA peptide, and M2e peptide were synthesized by VCPBIO Inc. China. Following are the location and amino acid sequences of the peptides NS1_34-49 (Biotin-DRLRRDQKSLR GRGNT), NS1_23-42 (Biotin-ADQELGDAPFL DRLRRDQKS), NS1_87-98 (Biotin-TDMTLEEM SRDW), NS1_221-233 (Biotin-QKRKMARTIESEV), HA_488-516 (Biotin-DYPQYSEEARLKREEISG VKLESIGIYQI), M2e (Biotin-MSLLTEVETPTR NEWECRCSDSSD). All peptides, which were biotinylated at the N-terminal, had at least 90% purity.

Serum

Sera used in this study were collected from chicken experimentally infected with H5N1 influenza virus (A/Chicken/WestJava/Sbg-29/2007). The challenge experiment had been described in our previous study (Tarigan et al. 2015). Briefly, 200 layer chicken were divided into 4 groups (A, B, D and C). Groups A, B, and D were vaccinated with a commercial-killed-H5N1 vaccine 3, 2 and 1 times, respectively. Group C birds were not vaccinated and served as a control. Two weeks after the last vaccination, randomly selected birds from each groups including the control were challenged with an isolate of H5N1 virus. For the current experiment, 18 sera from each group of pre-vaccination, post vaccination, 1, 2-3, and ≥4 weeks post challenge were selected.

Direct-peptided- and streptavidin-peptided-coated ELISA

Two types of ELISA were used based on the method used for coating the peptides on the 96-well microtitre plate. In the first ELISA, each biotinylated peptide was disolved in carbonate buffer (pH 9.6) at 5

µg/ml then added to the plates at 100µl/well). After leaving at 4°C overnight, the plate was blocked with 5 mg/ml of non-fat-skim milk in PBS (pH 7.2), 150 µl/well for 2 hours. Serum diluted at 1:100 in PBST(PBS, 0.5% Tween 20) supplemented with 5% normal rabbit serum was added at 100 µl/well, then incubated at 37°C for 2 hours. In each plate, negative control SPF chicken serum was added. After washing four times with PBST, HRP-rabbit-anti-chicken IgG conjugate (Sigma Co.) diluted 1:5000 in PBST supplemented with 5% normal rabbit serum was added, then incubated at 37°C for 2 hours. After washing four times with PBST, solution of substrate and chromogenic ABTS was added and the absorbance (A_{420}) measured with a spectrophotometer microtitre plate reader.

In the second type of ELISA, streptavidin disolved in carbonate buffer at 4 µg/ml, was added 100 µl/well, and left overnght at 4°C. Streptavidin used in this study was produced in the previous study (Tarigan & Sumarningsih 2014). After washing four times with PBST, biotinylated peptide diluted in PBS at 5 µg/ml was added at 100 µl/well. The plate was blocked with skim milk and the remining procedures were the same as in the first type of ELISA.

A preliminary assay was carried out to prove that streptavidin coated to the plates did specifically bind to the biotin, and to determine the optimum concentration of streptavidin for the assay. Streptavidin was diluted to 4, 2, 1, 0.5, 0µg/ml in carbonate buffer and added to mocrotitre plate at 100 µl/well. After leaving at 4°C overnight, the plate was blocked with skim milk for 2 hours. Biotynilated chicken IgY, produced in the previus study (Tarigan et al. 2015), was added at 1:1600 and 1:3200 dilution in PBS and incubated at 37°C for 2 hours. After washing four times with PBST, HRP-anti chicken IgG (Sigma Co.) at 1: 5000 dilution was added and incubated at 37°C for 2 hours. After washing four times with PBST, solution of substrate and chromogenic ABTS was added and the absorbance (A_{420}) measured with a spectrophotometer microtitre plate reader.

Statistical analysis

The level of antibody, indicated by ELISA's OD, to each peptide in each group of bird (pre-vaccination, post-vaccination, 1-week-post challenge, 2-3-week-post challenge, and ≥4-week-post challenge) was presented as the mean and 95% confidence interval of the mean of the ELISA's OD. Differences between groups were analised by one-way ANOVA (analysis of variance). All statistical analyses were carried out using a commercial statistical package (IBM® SPSS® Statistics).

RESULTS AND DISCUSSION

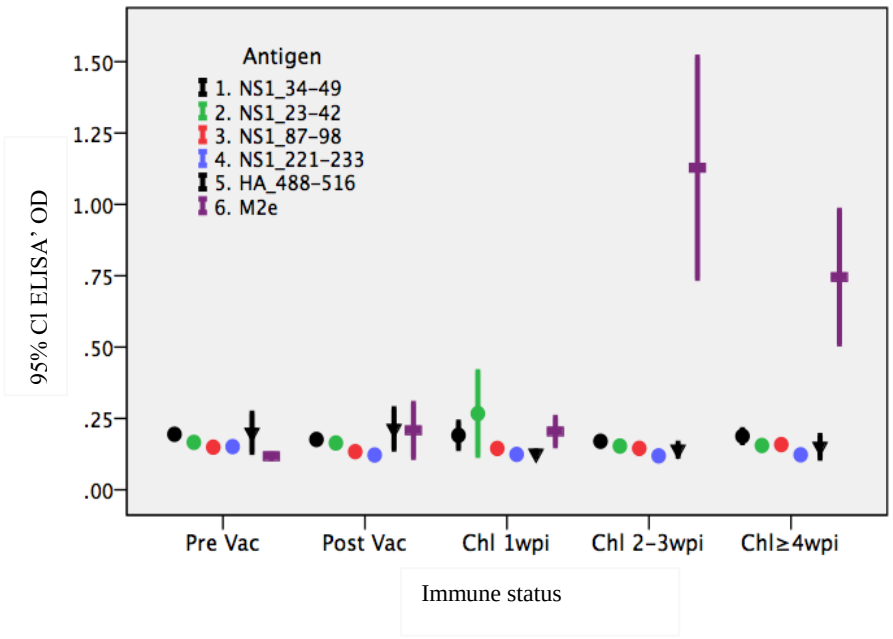
Results

Antibodies to NS1, HA_488-516 and M2e, measured by direct peptide ELISA in chickens of various immune or infection status, are presented in Figure 1. No antibody to any of the four NS1 peptides, the HA_488-516 peptide or M2e peptide was detected in chickens before and after vaccination. Antibody to M2e rose markedly after 1 week and remained high for several weeks after challenge. Unlike antibody to M2e, no antibody conversion was observed to any of the NS1 or HA_488-516 peptides.

There are two possibilities regarding the lack of measurable antibody conversion to the NS1 and HA2 peptides. The first possibility was that antibodies did not develop in those infected birds. This possibility seemed unlikely because antibody to NS1 should be detected whenever replication of influenza virus take place in immuno-competent animals. The strong antibody response to M2e indicated that a substantial replication of H5N1 virus must have had taken place in those infected birds. The second possibility was that the direct peptide ELISA used in this study was not sensitive enough to detect the presence of NS1 or HA_488-516 antibodies. Onepossible cause of this insensitivity was that the NS1 and HA2 peptides did not bind to the plates, or were unable to bind the antibodies once the peptides were immobilised on the plates. This problem was aliviated by the use of the streptavidin-peptide ELISA.

When added to the streptavidin-coated, protein-blocked plates, biotinyl-IgY was bound to the plate efficiently. In contrast, no biotinyl-IgY was retained when added to a non-streptavidin-coated, protein-blocked plates (Figure 2). This results indicated that the binding activity of streptavidin coating was specific and efficient. The concentration of streptavidin on coated plates that maximally bound the biotinyl IgY was around 4 µg/ml, and this concentration was used in all assays in this study. When the streptavidin-peptide ELISA was used to assay the chicken sera, the results, regarding antibody to M2e, were comparable to the direct peptide ELISA described above.

This streptavidin-peptide ELISA, however, was able to show that the level of antibody to all peptides in the sera of infected birds were higher than those in the non-infected birds (Figure 3). Although the increase of antibodies were only slight, they were statistically significant ($P>0.05$). The statistically significant increases or differences were between pre-vaccinated and challenged (≥4 wpi), and post-vaccinated and challenged (≥4 wpi) sera, to all peptides (Table 1).



Pre Vac = Pre vaccination;
Post Vac = 2 week post vaccination (1, 2 and 3 times vaccination);
Chl 1 wpi = 1 week post challenge;
Chl 2-3 wpi = 2 and 3 weeks post challenge;
Chl ≥ 4wpi = 4 week or longer after infection or challenge

Figure 1. Antibody conversions to four NS1, HA_488-516 and M2e peptides in chickens after vaccination and challenge with H5N1 virus

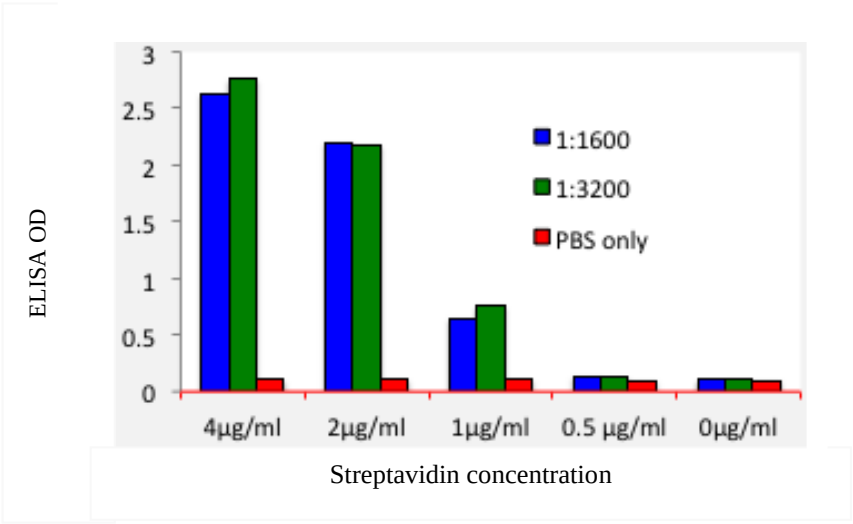
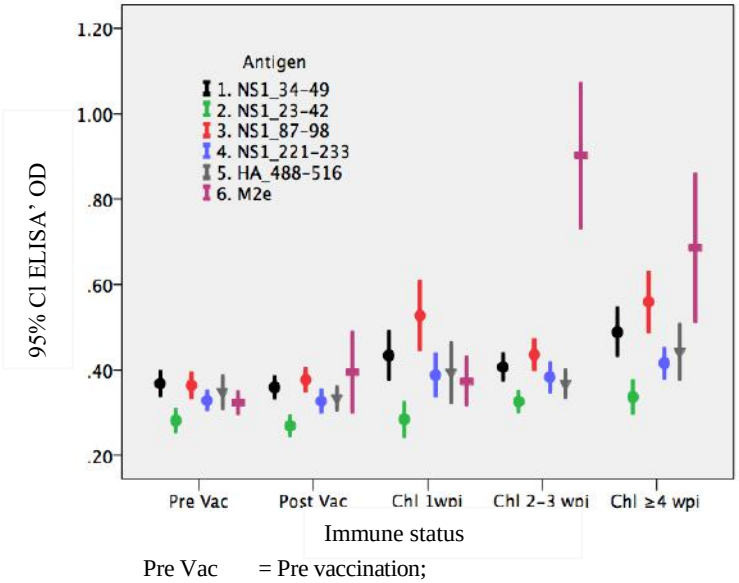


Figure 2. Binding of biotinylated IgY to streptavidin coated onto wells of microtitre plate. Biotinylated IgY was added to streptavidin-coated, protein-blocked microtitreplate. The biotinylated IgY bound to streptavidin was detected by HRP-anti-chicken IgG and the quantity of biotinylated IgY bound to the strepatvidin was correlated with the ELISA's OD



Pre Vac = Pre vaccination;
Post Vac = 2 week post vaccination (1, 2 and 3 times vaccination);
Chl 1 wpc = 1 week post challenge;
Chl 2-3 wpc= 2 and 3 weeks post challenge;
Chl ≥4 wpc = Post challenge 4 week or longer

Figure 3. Antibody conversions to NS1, HA2 and M2e peptides in chicken after vaccination and challenge with H5N1 virus

Table 1. Antibody to NS1 (NS1_23-42, NS1_34-49, NS1_87-98, NS1_221-233), HA2 and M2e peptides in chickens that increased significantly (P<0.05) after challenge with H5N1 ELISA streptavidin

		1 week post challenge	2 and 3 weeks post challenge	Post challenge 4 week or longer
NS1_23-42	Pre vaccination		✓	✓
	2 week post vaccination (1, 2 and 3 times vaccination)		✓	✓
	1 week post challenge			
	2 and 3 weeks post challenge			
NS1_34-49	Pre vaccination			✓
	2 week post vaccination (1, 2 and 3 times vaccination)			✓
	1 week post challenge			
	2 and 3 weeks post challenge			
NS1_87-98	Pre vaccination	✓	✓	✓
	2 week post vaccination (1, 2 and 3 times vaccination)	✓		✓
	1 week post challenge			
	2 and 3 weeks post challenge			✓
NS1_221-233	Pre vaccination		✓	✓
	2 week post vaccination (1, 2 and 3 times vaccination)			✓
	1 week post challenge			
	2 and 3 weeks post challenge			
HA_488-516	Pre vaccination			✓
	2 week post vaccination (1, 2 and 3 times vaccination)			✓
	1 week post challenge			
	2 and 3 weeks post challenge			
M2e	Pre vaccination		✓	✓
	2 week post vaccination (1, 2 and 3 times vaccination)		✓	✓
	1 week post challenge		✓	✓
	2 and 3 weeks post challenge			

✓ = Antibody (ELISA OD) was significantly increased (P<0.05)

No differences in all antibodies between pre and post-vaccinated sera. The level of antibody to NS1_87-98 peptide was significantly higher in the 1-week-post-challenge sera than in pre- and post-vaccinated sera. The higher level at the 1-week-post-challenge sera was not observed in antibody to other peptides, even that to M2e.

Discussions

This study shows that M2e-based ELISA is superior to NS1- or HA2-based ELISA as DIVA test. As far as we are aware, no study has been carried out previously comparing M2e ELISA and NS1-peptide-based ELISA as DIVA test.

Influenza-NS1 protein consists of 230 amino acids and two functional domains: the RNA-binding (aa residues 1-73) and the effector domain (aa residues 74-230). The RNA binding domain funtions to inhibit action of interferon (Donelan et al. 2003; Min & Krug 2006). The effector domain functions to enhance viral mRNA translation, deregulate cellular mRNA processing, inhibit dsRNA-activated protein kinase and activate phosphoinositide 3-kinase (PI3K) signaling (Hale et al. 2008).

The NS1 protein is the only true non-structural protein in influenza viruses (Donelan et al. 2003). Being the non-structural protein, it is expressed only during viral replication inside the host cells but the protein is not packed into the virion. Driven by the assumption that animals vaccinated with killed AIV vaccines do not develop specific antibodies to the NS1 protein, as do animals infected by life virus, interest in developing a DIVA test based on the NS1 protein has been long present. However, there is still disagreement between different studies on the accuracy of NS1-based ELISA used as DIVA test. Most studies showed that NS1-based tests had low accuracy as a DIVA test, as was found in this present studies (Tumpey et al. 2005; Dundon & Capua 2009; Avellaneda et al. 2010). Other studies, however, indicated that NS1-based ELISA ispotentially useful as DIVA test (Birch-Machin et al. 1997; Ozaki et al. 2001; Tumpey et al. 2005; Takeyama et al. 2011; Wang et al. 2011; Golchinfar et al. 2014). In spite of that potential of NS1-based ELISA as DIVA test, the test had rather low specificity because some vaccinated animals were also seropositive although with low antibody titres. The NS1 antibodies are presumed to be induced by the NS1 protein derived from cellular debris of chicken embryo contaminating the vaccines (Tumpey et al. 2005). The NS1 ELISA has not only low specificity but also low sensitivity. The low sensitivity is associated with the nature of the protein that is poorly immunogenic. The NS1 antibody is usually in low titre and rapidly disappears (Tumpey et al. 2005; Avellaneda et al. 2010). Immune response to NS1 may also be species-dependent. An experimental study reported that infection of chicken with a LPAI isolate caused antibody conversion to NS1 protein only in 3 of 14 birds, and the antibody was detected only at day-3 post infection. Infection of turkey with the same isolate produced higher proportion of seroconversion and the antibody was detected at day-5 to day-10 post infection (Dundon & Capua 2009).

There are several possible causes of the NS1-antibody undetectable in infected chicken. The first possible cause is that the assay used is not sensitive enough to detect the presence of the antibodies. In this present study, four synthetic peptides with amino acid sequence based on the regions of the protein considered to be immunogenic based on hydrophobic analysis. A peptide comparable to that NS1_34-49 in this study has previously been demonstrated to be sensitive when used as coating in ELISA in detecting NS1 antibody (Tumpey et al. 2005). Test based on peptide NS1_23-42 that proceeds at the N-terminal, and overlaps 9 amino acids with NS1_34-49 also failed to detect the presence of NS1 antibody. As a matter of fact, Tumpey et al. 2005 shows that the peptide is more specific, although less sensitive, than the recombinant–whole-NS1 protein. This finding lend support to the opinion that ELISA based on the whole-NS1 protein is not necessary more accurate than that bases on NS1-synthetic peptide in detecting the antibody to the NS1 protein.

The C terminal end of NS1 protein that contain the PDZ-ligand binding motif (PBM), ESEV, has been proven to be immunogenic, and animals infected by influenza viruses become seroconverted to synthetic peptides which sequence is based on the C terminal part of the NS1 protein (Birch-Machin et al. 1997; Dundon et al. 2006). In this present study, however, the sera from infected birds did not contain antibody to a comparable peptide, NS1_221-233, assayed with the direct-peptide coating ELISA.

Sera from human survived from H5N1-virus infection have been shown to contain antibody to a HA2 peptide, HA_488-516 (Khurana et al. 2011). The antibody reported to be specific for H5N1-virus infection as it was not detected in sera from human vaccinated or infected with seasonal influenza virus, or vaccinated with subunit H5N1 vaccine (Khurana et al. 2011). These results suggest that the peptide deem a good candidate for DIVA test in poultry. However, our study showed that the infected birds failed to be positively converted to the same HA_488-516 peptide.

It is not only the peptides themselves, the ELISA format, the direct coatingof peptides onto the wells of ELISA plates, used in this study was also similar to the previous studies (Tumpey et al. 2005; Dundon et al. 2006). Therefore, the probability that the peptides were notimmobilised on the microtitre plate as the cause of the failure to detect the NS1 antibody in sera of

challenged birds in our study seems unlikely. This possibility was partially ruled out by the streptavidin-peptide ELISA. The streptavidin coated onto the plates has been shown to specifically bind to biotinyl substances. Since the peptides used were all biotinylated at the N terminal, the peptide were expected tobind to the plate only through its N-terminal 'tip'; consequently, if asample serum contains NS1 antibody, it should react with the immobilised peptide efficiently. The fact that the ELISA's ODs of challenged sera increased significantly as compared to those before challenge indicated that the streptavidin-peptide coated ELISA is more sensitive than the direct-peptide coated ELISA. Since the increased were only slight even in the improved assay, it indicates that the conversion of antibody to NS1 protein in the infected birds is only mild and only detected by a very sensitive assay.

In summary, this study shows that antibody to either M2e, HA_488-516, NS1 peptides, are absent in bird naïve or vaccinated with killed H5N1 vaccines. Antibody conversion to M2e is more consistent and its magnitude is much higher than that to HA_488-516 and peptide NS1 peptides. Consequently, M2e-based is much more reliable than NS1- or HA2-based ELISA as DIVA test. Antibody conversion to HA_488-516 and peptide NS1 in infected chickens is extremely mild and therefore difficult to detect.

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Newcastle Disease Virus Infection Study on Duck and Chicken in Subang District

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ABSTRAK

Panus A, Setiyaningsih S, Mayasari NLPI. 2015. Kajian infeksi virus *Newcastle Disease* pada bebek dan ayam di Kabupaten Subang. JITV 20(2): 134-147. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1168

Tujuan dari penelitian ini adalah untuk mendeteksi dan mengetahui keragaman antigenik virus Newcastle disease (NDV) yang bersirkulasi di Kabupaten Subang. Sampel usapan kloaka, usapan orofaring dan serum diambil dari 393 ekor ayam dan 149 bebek dari penampungan, peternakan dan pasar unggas di 10 kecamatan di Kabupaten Subang. Screening NDV pada sampel pool (5-7 individu per pool) dengan real-time Reverse-Transcription Polymerase Chain Reaction (rRT-PCR) matrix (M) menunjukkan 19 dari 67 pool kloaka (28,3%) dan 8 dari 67 pool orofaring (11,9%) ayam terdeteksi NDV; 18 pool dari 67 pool ayam (26,9%) menunjukkan virus diekskresikan melalui kloaka dan orofaring. Sementara pada sampel itik, NDV terdeteksi hanya pada kloaka yaitu 8 dari 30 pool (26,7%). Total 18 isolat berhasil diisolasi dari sampel usapan kloaka dan usapan orofaring individu yang menunjukkan karakter antigenik yang homogen, namun beberapa isolat menunjukkan variasi dengan titre sampai 2 Log2 menggunakan antisera LaSota dan 2-4 Log2 dengan antisera Komarov. Mayoritas isolat menunjukkan afinitas lebih tinggi terhadap antisera Komarov, yang mengindikasikan semua isolat adalah NDV galur ganas. Karakterisasi patogenisitas dengan uji elusi hasilnya menunjukkan 3 isolat masuk ke kelompok galur mesogenik dan 15 isolat ke kelompok galur velogenik, sedangkan dengan rRT-PCR fusion (F) menunjukkan 100% isolat merupakan galur ganas (mesogenik/velogenik). Deteksi antibodi spesifik terhadap NDV pada 408 serum dengan uji HI menunjukkan 48 serum (12%) positif dengan kisaran titre 1 sampai 8 Log2; hanya sekitar 13% ayam yang divaksin menunjukkan titre protektif (≥3 Log2). *Newcastle disease* masih endemik di Kabupaten Subang dengan variasi antigenik galur virus yang bersirkulasi relatif tidak telalu bervariasi.

Kata Kunci: Newcastle Disease, rRT-PCR, Virulensi, Keragaman Antigenik, Antibodi

ABSTRACT

Panus A, Setiyaningsih S, Mayasari NLPI. 2015. Newcastle Disease Virus infection study on duck and chicken in Subang district. JITV 20(2): 56-69 DOI:http://dx.doi.org/10.14334/jitv.v20i2.1168

The objectives of this research were to study Newcastle Disease Virus (NDV) infection in Subang area and to examine the diversity of the circulating NDV. Swabs of cloacal and oropharynx, and serum were sampled from total of 393 chickens and 149 ducks in backyard farms and live bird markets located in 10 subdistricts. Screening of NDV in pool of 5-7 samples by real-time Reverse-Transcription Polymerase Chain Reaction (rRT-PCR) matrix (M) showed 19/67 (28.3%) cloacal and 8/67 (11.9%) pharyngeal pools of chicken samples; 18/67 (26.9%) of the pools excreted virus via cloaca and oropharynx, while the duck pools of 8/30 (26.7%) shed virus from cloaca. Virus isolation attempted on individual sample from positive pools yielded 18 isolates which the majority of the isolates showed homogeneous antigenic character, only some of these showed variations up to 2 Log2 with Lasota and 4 Log2 with Komarov antisera. Majority of isolates had a higher affinity to Komarov indicating their propencity to virulent strains. Pathogenicity examination using elution test showed 3 isolates virus were grouped to mesogenic strains and 15 isolates to velogenic strain, in agreement with rRT-PCR fusion results. HI test on 408 sera showed that NDV antibody was detected in 48 (12%) birds with titres ranging from 1 to 8 Log2; only about 13% of vaccinated chickens demonstrated protective antibody titre (≥3 Log2). Newcastle disease is still endemic in Subang with relatively low antigenic variation among circulating strains.

Key Words: Newcastle Disease, rRT-PCR Detection, Detection of Virulence, Antigenic Diversity, Antibody

INTRODUCTION

Newcastle Disease (ND) is one of serious diseases in poultry because it is very contagious, spread rapidly and attack some species of birds at all age. Mostly this outbreak attacks intensive poultry as follows: chicken, turkey, duck, quail, and pigeons. ND spread all over the world and potentially causes economy losses in poultry industry. In addition of poultry, this disease infect and causes death in wild birds as well. ND cases were firstly found and reported in the mid of 1920 in Indonesia (Java Island) and England (OIE 2012), then spreading in a few years later and becoming endemic in many countries (Ashraf & Shah 2014). Nowadays, almost all regions in Indonesia are affected and no one area or island is free from ND. In spite of mortality rate caused by ND was controlable, the effect in production is still a problem. Moreover, the impact of other losses is the costs for controlling the disease and also stopping export from ND endemic countries (Brown et al. 1999).

The outbreak caused by ND can be acute or chronic and infecting all species of birds especially chicken, both domestic and purebred. The outbreak occured in the field may caused by various strain of ND virus. According to the severity-level of the outbreak in chicken, Newcastle Disease Virus (NDV) was classified into three pathotypes namely lentogenic, mesogenic, and velogenic. Velogenic strain is distinguished into neurotropic and viscerotropic form (Aldous & Alexander 2001).

The loss caused by ND are morbidity and mortality which in infected poultry the rate may reach 100% caused by velogenic strain especially in sensitive chicken groups and under 10% in mesogenic strain (OIE 2008). In the developing countries where the livestock industry is growing very rapidly, the losses affected by NDV outbreak are not only mortality but also expenditure additionaly cost used for vaccination, biosecurity and depopulation. Even the free ND countries, have to spend on periodic testing in order to maintain free status from ND which needed for trading license. Moreover, in the developing countries as endemic ND, the impacts are not only economy losses but also affecting health and socioeconomic condition of lower-class society, whose quality and quantity of eggs and meat consumed decreased caused by ND (Alexander & Senne 2008). In 2002, ND outbreak in California, United States caused losses 200.000.000 US$ as an impact of depopulation (Kapczynski & King 2005). The losses affected by ND in layer are mortality and reduction of egg production, while causing growth disorder and reduction of body weight in boiler. Data of OIE (2009) showed in 2007, about 1500-8000 chickens were infected by ND every month in Indonesia. Moreover, according to Xiao (2012) in 2009 and 2010, ND outbreak occured in comercial chicken in Indonesia causing 70-80% mortality. ND is still become a major problem in the poultry industry despite the vaccination carried out routinely (Samal 2011). Therefore, ND is a serious threat for poultry in Indonesia. Subang area in West Java is one of buffer zones of poultry production, particularly for broilers and layers. Totally 44.049.739 poultry population was reported in 2013 (DISNAK 2013). Newcastle Disease is endemic in Indonesia including in Subang area. DISNAK (2013) recorded, there were 258 birds suddently died caused of ND infection in 2010 and it was confirmed by rRT-PCR using cloacal and oropharyngeal swabs and organ samples. Mass dead might occur if it was not handled properly. Annually survey by Balai Penyidikan dan Pengujian Veteriner (BPPV) Subang in unvaccinated ND backyard birds in 2011, found 10 out of 131 serums tested were positive of ND with titre range 2-5 Log2 (BPPV 2011). In 2012, 12 out of 37 serums tested were positive of ND with titre range 1-4 Log2 (BPPV 2012), and in 2013, 184 out of 359 serums tested were positive of ND with titre range 2-8 Log2 (BPPV 2013). These results show that ND is still endemic in Subang area. As the basis of consideration for efective control measures and prevention, it is neccesary to conduct NDV isolation and detection of antibody against ND in ducks and chickens in Subang area.

For the time being, investigation of ND in Subang area is still limited. Commonly, the diagnosis was based on clinical symptoms, pathological alteration and serological test. Therefore, diagnostic technique with high sensitivity to detect and confirm NDV infection in ducks and chickens in Subang area is required.

MATERIALS AND METHODS

Samples

Samples were taken from 10 subdistricts in Subang, that were Binong, Ciasem, Cipendeuy, Cipunagara, Compreng, Pagaden, Pusaka Nagara, Subang, Sukasari and Tambak Dahan. These areas were selected because population of fowls were centralized in those locations (market, shelter, farm) and endemic area of ND as well.

Standard antigen and antisera and Kit

ND virus standard (4HAU) LaSota strain (collection of FKH IPB), specific standard antisera against LaSota and Komarov strain were used for HI test (collection of BBPMSOH). QIAamp® Viral RNA Mini Kit (Qiagen) was used for RNA virus extraction. Ag-Path ID™ One-Step RT-PCR kit from *Life Technologies* with 96 optical plates in *Applied Biosystems 7500 Real Time PCR System Software Version* 1.4.0 were used for Real time RT-PCR.

Collection of swabs and serum samples

Swabs of cloacal and oropharynx were taken from chickens and ducks from the bird's shelter, livebirds market and poultry farms in 10 areas in Subang using sterile cotton swabs inserted in microtube 2 ml contains *Brain Heart Infusion Broth* (BHIB). The temperature was kept cool (4-8°C) until arrival in the laboratorium.

Pooling swabs of cloacal and oropharynx samples consist of 5-7 individual samples in each pool was based on swab types, birds, location and time of sampling. The sample pool was subsequently used for rRT-PCR test using *primer matrix* (M). Blood was collected via branchial vein from each individu along with swab samples.

real time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) Test

To detect the presence of genetic material of NDV extracted from swabs of cloacal and oropharynx, rRT-PCR test with NVSL protocol (2005) was done. RNA virus isolation was extracted based on *QIAamp® Viral RNA Mini Kit* (Qiagen) standard procedure. rRT-PCR amplification using *Ag-Path ID™ One-Step RT-PCR kit* from *Life Technologies* in *Applied Biosystems 7500 Real Time PCR System* was conducted. Cycles of rRT-PCR was performed in 45°C for 10 minutes, 95°C for 10 minutes, 95°C for 10 minutes, 56°C for 32 seconds, and 72°C for 10 seconds. The result was analyzed by *Applied Biosystems 7500 Real time* PCR *SystemSoftware Version* 1.4.0. Primer and probe were used are presented in Table 1.

Virus isolation in SPF embryonated chicken egg

Swabs of cloacal and oropharynx samples used as inoculum were from individual bird sample from positive rRT-PCR M pool. As much as 0.2 ml inoculum containing *penicillin-streptomycin* (9:1) and incubated for 30 minutes at ambient temperature (25-27°C) was injected in allantoic cavity of Specific Pathogen Free (SPF) embryonated chicken egg. Eggs were incubated in incubator at 37°C for 4-7 days and observed 3 times a day to check the viability of embryo (OIE 2012). Isolates obtained from alantoic liquid were reconfirmed with rRT-PCR matrix (M).

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Test

Procedures of HA and HI test was done by micro methods (OIE 2012), performed by adding 25 µl 0.85% *phosphate buffered saline* (PBS) into micro plate in 1st-12th pit using micro pipet. In the 1st pit 25 µl serum standard was added and diluted, than moved into 2nd-11th pit. A total of 25 µl 4HAU ND virus suspension was added into each 1st-10th and 12th pit, and then incubated at ambient temperatute for 15 minutes and 25 µl suspension of 1% red blood cells (rbc) was added into 1st-12th pit, homogenized and incubated at ambient temperature (25-27°C) for 40 minutes. Positive result marked by occurance of resistance hemagglutination in form of precipitation of rbc on the bottom of micro plate pit. Titre of HI was determined based on the highest serum dilution that was still showed precipitation (agglutination inhibition). HA and HI test were performed 3 times.

Elution-time test

Test was performed based on Ezeibe & Ndip (2005) procedures. A total of 50 µl PBS solution was put into micro plate pit, then 50 µl virus suspension was added into 1st pit, and diluted into 1st-10th pit. As much as 50 µl PBS was added into each 1st-12nd pit, followed by 50 µl suspension of 0.6% rbc into 1st-12nd pit, homogenized and incubated at ambient temperature for

Table 1. Primers and Probes used for rRT-PCR matrix (M) and fusion (F)

Target of gene	Primer/genom probe targets	Sequence(5'→3')	Reference
APMV-1 M (matrix)	M+4100 forward	AGTGATGTGCTCGGACCTTC	Wise et al. (2004)
	M+4169 probe matrix	[FAM]TTCTCTAGCAGTGGG ACA GCC TGC[TAMRA]	
	M-4220 reverse	CCT GAG GAG AGG CAT TTG CTA	
APMV-1	F+4829 forward	GGTGAGTCTATCCGGARGATACAAG	CVL (2007)
F (Fusion)	F+4939 reverse	AGCTGTTGCAACCCCAAG	
	F+4894 Probe 1 (Virulent)	[FAM]AAGCGTTTCTGTCTCCTTCCTCCA [TAMRA]	

40 minutes, then hemagglutination was observed. Elution time was determined based on time of complete hemagglutination was observed on highest dilution until the precipitation of rbc showed (elution). Elution-time test was performed 3 times.

Data analysis

Data were analyzed descriptively and statistically to determine standard mean deviation (SD). The average of antibody titre was calculated by geometric mean titre (GMT) by the formula:

$$\text{Log2 GMT} = \frac{(\text{Log2 t1})(\text{S1}) + (\text{Log2 t1})(\text{S1}) + \ldots + (\text{Log2tn})(\text{Sn})}{\text{N}}$$

Information:
- N = Number of observed serum
- T = Antibody titre at the highest dilution (which was still may inhibit agglutination of red blood cell)
- S = Number of titrated serum
- n = Antibody titre of the n^{th} sample

Coefficient of variation/CV from immune response was expressed by following formula:

$$CV = \frac{S}{\bar{X}} \times 100\%$$

Description:
- CV = Coefficient of Variant,
- S = Standard Deviation,
- $\bar{X}$ = Average of antibody titre

RESULT AND DISCUSSION

Detection of NDV in pool of swabs of cloacal and oropharynx with rRT-PCR matrix (M)

There were 542 samples succesfully collected consist of 149 ducks and 393 chickens sample comprised of 108 broilers, 148 broiler parent stocks, 15 layers and 122 lokal chickens from 10 areas in Subang district.

Testing for 97 pools swabs of cloacal resulted in 27 pools (29%) of positive which spreaded in 9 areas, 8 pools (7%) oropharynx swabs positive spreaded in 3 areas and 18 pools (18%) of cloacal and oropharynx swabs positive spreaded in 6 areas while none was positive from Cipendeuy area (Table 2). ND virus was only detected in cloacal swabs of ducks (8/30 pools), 19 pools cloacal swabs, 8 pools oropharynx swabs and 18 pools cloacal and oropharynx swabs (Table 1). Ducks tend to excrete the virus via cloaca according to the findings reported by Saepulloh & Darminto (2005) which were 14 (13%) isolates from cloacal and none from oropharynx swabs of 106 ducks in Kalimantan.

The highest number of positive M cloacal swabs pools (7) were obtained from Tambak Dahan area. The highest number of positive M oropharynx swabs pools (4) were obtained from Cipunagara area and the highest number of positive M cloacal and oropharynx swab pools (8) were obtained from Binong area, while in Cipendeuy area in pool of cloacal or oropharynx swabs, NDV were not detected.

Isolation of NDV in SPF embryonated chicken eggs

NDV virulence could be determined based on the infected embryo's death time. According to Cattoli et al. (2011), NDV causing embryo's death in more than 90 hours after inoculation was grouped in to lentogenyc strain, and between 60-90 hours grouped in to mesogenyc strain, while less than 60 hours, was grouped into velogenyc strain.

Inoculation of 128 cloacal swabs positive rRT-PCR M, 10 isolates were obtained, and from 76 positive oropharynx swabs, 8 isolates obtained. Totally, 18 NDV were succsessfully isolated and they were 3 isolates from 3 ducks and 15 isolates from chickens. The virus excreted via chicken's cloaca and oropharynx was balanced; 7 isolates from native chicken cloaca and oropharynx, 6 isolates from broiler oropharynx and 2 isolates from 2 native chicken oropharynx. Four out of 18 isolates were excreted from cloacal and oropharynx from 2 native chickens. In Binong area, pool of cloacal and oropharynx swabs sample were not contain NDV (negative rRT-PCR M) so isolates were not obtained from layer (Table 1). Transmission route of virus from the host body affected by tropism tissue of NDV. The virus which is replicated in respiratory tract will be shed through mouth and nostril and NDV replicated in digestive tract will be shed through cloaca. During incubation, virus replicated at the entry site. Virulent NDV strain (mesogenyc and velogenyc) could invaded into blood vessels, following the blood circulation and replicated in visceral organs, then excreted through the feces (Alexander & Senne 2008). The replication of virulent NDV strain in visceral organs causes tissue damage, such as lesions in brain, hemorrhage and necrosis of the intestinal tract, respiratory and caeca tonsils. Haemorrhage can be found in the craw, heart, skin and eyelids as well (Figure 1).

Table 2. The number of birds and pool sample from 10 subdistricts in Subang with rRT-PCR M test results and number of NDV isolates with antibody titre from individual of NDV detected

District	∑ Birds/*Pool*		∑ *pool* matrix with the result (+)			∑ Isolate was obtained	Type of sample
			Duck/Chicken				
	Duck	Chicken	C	O	C&O	Duck/Chicken	
Ciasem	22/4	2 Kp/1	1/1	0/0	0/0	1/0	C
Pusaka Negara	24/5	-	2/0	0/0	0/0	1/0	C
Sukasari	22/5	-	5/0	0/0	0/0	1/0	C
Tambak Dahan	31/7	57 Kp /12	0/6	0/1	0/1	0/2 Kp	2C
Binong	-	15 L /3	0/0	0/0	0/0	0/0	-
	-	86 Br /13	0/3	0/3	0/8	0/4 Br	4O
Compreng	-	20 Kp /4	0/1	0/0	0/1	0/2 Kp	1C & 1O
Pagaden	-	15 Kp /3	0/1	0/0	0/1	0/3 Kp	1C, 1C&1O
Cipunagara	23/4	18 Br/3	0/0	0/0	0/0	0/0	-
	-	153 Br. PS /22	0/4	0/4	0/5	0/2 Br.PS	2O
Cipeundeuy	27/5	-	0/0	0/0	0/0	0/0	-
Subang	-	27 Kp /6	0/3	0/0	0/2	0/2 Kp	2C
Total	149/30	393 / 67	8/19	0/8	0/18	3/15	

Kp= kampong; L= layer; Br= broiler; PS= parent stock; C= cloaca; O= oropharynx

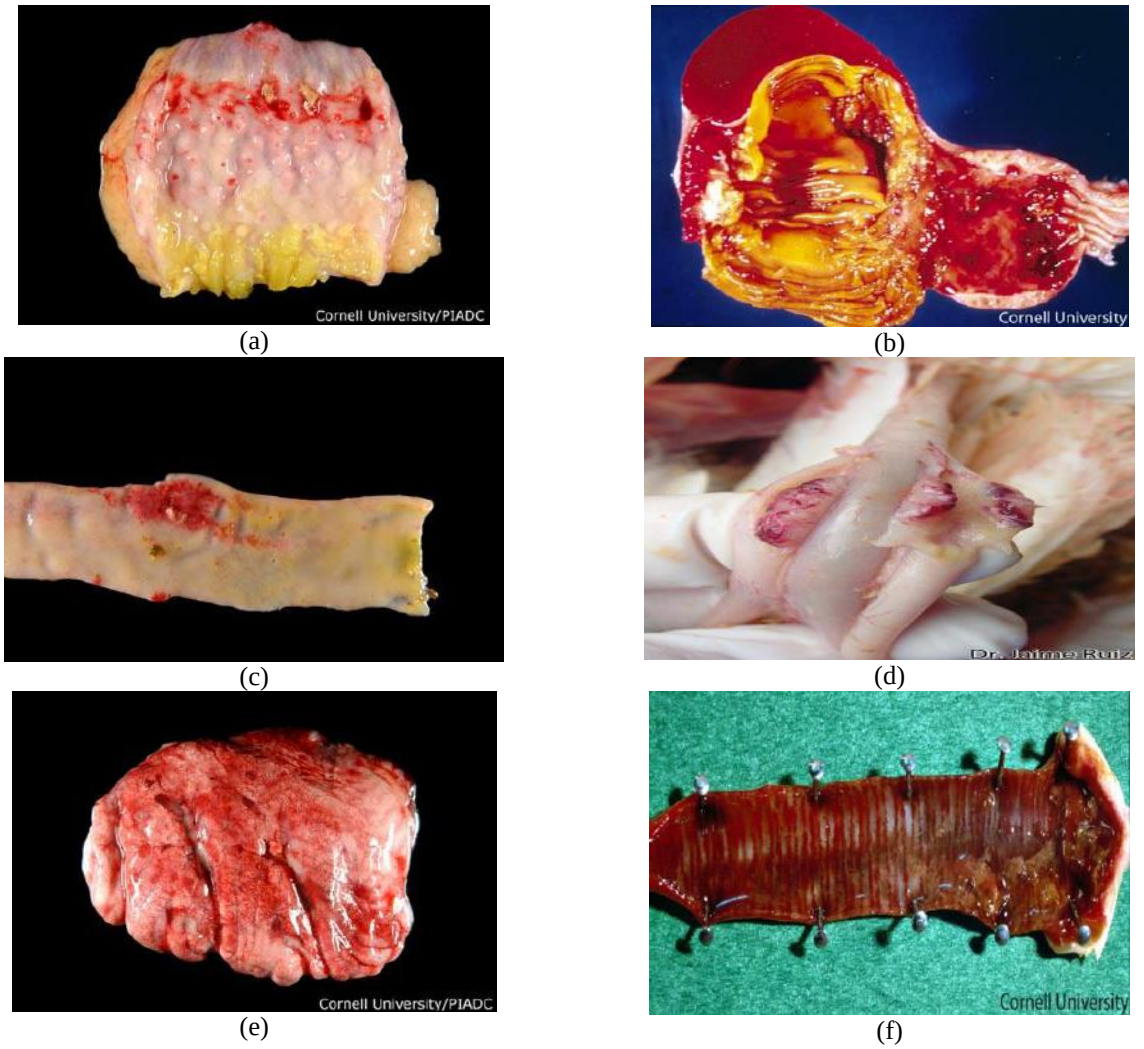
According to Pertulla (2009) percentage of mortality due to infection of velogenyc NDV strain could reached 90% and usually the infected birds will die in 1-2 days after infection. Isolation NDV from swabs of bird cloacal and oropharynx samples from the field in Bangladesh have been done by Haque at al. (2010), and the result showed that 18 isolates were obtained from 20 cloacal swabs and 17 isolates from 20 oropharynx swabs.

ND virus found in unvaccinated native duck was originated from natural infection and usually mesogenyc or velogenyc NDV strain infection in duck showed no clinical symptoms (Saepulloh & Darminto 2005). The NDV isolated from unvaccinated native chickens was also from natural infection. Detection of NDV with RT-PCR has been performed by Kencana et al. (2012) from 10 native chickens in acute-field case with short incubation period (1-2 days) reported and the result showed ND positive. Additionally, Adi et al. (2010) also succed in isolating velogenic NDV strain from native chicken when ND outbreak occur in Bali, and stated that keeping of free-range chicken tends to increase the opportunities for contact with other poultry which has potential risk in the transmission of ND. NDV also found in broilers and parent stocks which had been vaccinated. The chickens were still infected by ND even though it had been vaccinated. It seemed that the vaccination had been carried out was less effective. According to Dortmans et al. (2012), the main cause of the failure of vaccination is caused by improper vaccination implementation and also vaccinated chickens with low antibody usually vulnerable to NDV infection. While according to Hu et al. (2011), the NDV still found in vaccinated chickens because of the shedding of vaccine virus which not homologous with field virus. This was evidenced by Miller et al. (2013) experimentally by vaccinating chickens with LaSota vaccine, and then the chickens were challenged with heterologous virulent NDV (CA/2002) which different genotype with the vaccine virus and the result showed the challenge virus was still excreted into environment and infect unvaccinated chickens (control). According to Miller et al. (2013) using homologous vaccine with field virus may decrease excretion of virus into

environment more than heterologous vaccine. Most of ND vaccine not prevent vaccinated birds from virulent NDV infection but vaccination significantly may decrease the amount of virus excreted through saliva and feces compared to unvaccinated birds (Kapczynski & King 2005; Miller et al. 2009).

NDV was successfully isolated from ducks and chickens showed with and without illness symptoms. According to Emilia (2013), virus was found in the sample from the birds that did not show clinical symptom, possibly due to effect of partial infection in birds, so clinical symptom did not appear, however the virus still excreted. Saepulloh & Darminto (2005) stated that if the NDV can be detected in sick bird feces (cloaca), then this indicated of systemic infection.

Only 18 isolates successfully isolated from 204 (9%) swabs of cloacal and oropharinx that positive of M and inoculated into embryonated chicken eggs (Table 1), it was due to a lot of NDV did not multiplicate in eggs because of the virus already inactive due to the handling and transport of samples were unfavorable. A similar incident also occured in Emilia (2013) study, from 20 samples of individual that positive of gen matrix (M) which were inoculated in eggs, only 11 isolates were successfully isolated. This showed that the tRT-PCR test may detect inactive virus, according to Indriani et al. (2014) one of the advantages of rRT-PCR is able to detect genetic materials of virus either active or inactive. Detection of Antibody with Hemagglutination Inhibition Test (HI).



(a) (b) (c) (d) (e) (f)

Figure 1. Patognomonis pathological alteration of Newcastle Disease infection. *Ptechie* in proventriculus (a); ventriculus (b); intestine (c); caeca tonsil (d); oropharhynx (e); and lung (f) (Buckles et al. 2005)

HI test is often used in laboratories to examine specific antibody titres against NDV because its more specific and does not require special equipment so more economic (Syukron et al. 2013). The serums tested were selected based on representation of the flocks which detected positive and negative of NDV with rRT-PCR from each area. Distribution of antibody classified into three, namely: group 0, meaning that antibodies were not detected, <3, meaning low and no protective antibodies, and ≥3 groups, meaning that protective antibodies (Boven et al. 2008; Rezaeianzadeh et al. 2011).

HI test result on 403 serums from 10 areas showed that 48 serums (12%) containing varied titre antibody; 37 with ≥3 Log2 titre and 11 with <3 Log2 titre (Table 3). Antibodies against NDV detected in waterfowl (ducks), in the chickens were not vaccinated, and in vaccinated domestic birds. Antibodies against NDV detected in 9 of 18 duck serums from Pusaka Nagara area tested the spreading of 8 serums with ≥3 log2 titre and 1 serum with <3 Log2 titre, at 1 of 17 ducks serum and 1 of 32 chicken serums tested from Tambak dahan area with each ≥3 Log2 titre, at 10 of 62 broiler serums from Binong area tested with the spread of 5 serums with ≥3 log 2 titre and 5 serums with <3 log2 titre. in 1 of 13 chicken serum from Pagaden area tested with ≥3 log2 titre, in 26 of 135 broiler breeders (parents stock) serum from Cipunagara area tested with the spreading of 21 with ≥3 log2 titre and 5 with <3 log2 titres. Sample of cloacal and oropharyngeal swab and serum taken from a total of 27 ducks from Cipeundeuy area not found for NDV and content of specific antibodies against ND. This shows that duck samples from Cipeundeuy area were free from NDV. However, to ascertain whether the Cipeundeuy area is free of ND, it is necessary to do the detection and isolation of NDV and detection of specific antibodies against NDV in ducks and other birds such as native chicken and purebred chicken from other locations in Cipeundeuy area.

The percentage of total chicken serum that positively detected specific antibody against NDV (17%) was higher than the waterfowl (10%) and native chicken (2%). In vaccinated broilers, out of 212 serums tested only 36 serums containing antibodies against NDV, which 10 of them (28%) showed a low titre. This is because the serums were taken from culled broiler breeders which was not re-vaccinated of ND (live and killed vaccine) so antibody titre had been decreased, besides the serums were taken from broilers which had just only once ND vaccination and performed at day old with spray method using live ND vaccine and was not re-vaccinated (booster), so the possibility of uneveness of the titres is caused of antibody began to decline.

In waterfowl, only 3 isolates were successfully obtained from 8% positive of M cloacal pools and no isolates obtained from 1% positive of M oropharynx pools. In native chicken, it were 7 isolates obtained from 17% positive of M cloacal pools and 2 isolates obtained from 16 positive of M oropharinx pools. In broiler, there was not isolate obtained from 20% positive of M cloacal pools and 6 isolates obtained from 19% positive of M oropharinx pools (Table 3). There were not a lot of isolates of NDV were obtained, even there was some that not successfully obtained from positive of rRT-PCR M cloacal and oropharinx pools from flocks were also positively detected antibodies against NDV.

Value of coefficient of variation (CV) may used to describe the distribution of antibody titres in groups of animals. Mean and distribution of antibody titres in broilers using Geometric Mean Titre (GMT) and Coefficient of Varian (CV) calculations may be seen in Table 3.

Examination of 212 serum samples of native chicken from Binong and Cipunagara area showed that the average titres were low, ranging from 0-2.8. CV value of the lowest antibody titres was seen in flock of broiler chickens aged 3 weeks from the Binong area. It was 45.3% and the highest seen in culled PS broiler chickens flocks in Cipunagara. It was 185%, while the layer chicken flocks and broiler flocks from the Binong area and in broiler flock in Cipunagara area showed the mean titre of 0 and titres distribution of 0, due to the antibodies in the serum was not detected. CV value of antibody titres from broiler flock in Binong area (45.3%) and broiler flock in Cipunagara area (185% and 49.9%) were look ≥35%. This shows that distribution of antibody titres uneven well. The results of the average titre and distribution of antibody titres of vaccinated broilers in Binong and Cipunagara area were low and did not spreading well. It showed that vaccination of ND in broilers in both of Binong and Cipunagara subdistricts were not optimal.

HI titre showed the immunity status of bird. In unvaccinated native bird and did not show illness symptom, the existence of antibody indicated that the bird had been ever exposed by NDV (Alexander et al. 2004). In vaccinated birds with antibody titre at ≥3 Log2 level indicated a protective antibody. Herd immunity in a population is very important to be protected from the NDV. According to Boven et al. (2008) herd immunity will be obtained if 85% or more of antibody titre was at ≥3 Log2 level after twice vaccination. Kapczynski & King (2005) reported a field case which showed that only birds with anti body titre at ≥4 Log2 level and flock which has group immunity at 66% minimal which resistant to virulent NDV infection after many times vaccination. Generally, antibody titre ≥5 Log2 was considered the most protective. A phenomenon where flock of birds with high antibody titre is still may infected by virulent NDV or opposite of

Table 3. The result of detection antibody specific against NDV in bird serums from 10 areas in Subang area according to poultry commodities

Type/commodites	% Pool M +		Isolate		Programe Vaccination	∑ Serum	∑ +	% +	Antibody titre		
										Log2	
	C	O	C	O		Tested			0	<3	≥ 3
Waterfowl	8	1	3	-	No	102	10	10	92	1	9
Native chicken*	17	6	7	2	No	94	2	2	92	-	2
Purebred chicken	20	19	-	6	Yes	212	36	17	176	10	26
Total	45	26	10			408	48	12	360	11	37

C= cloacal; O= oropharynx; *the native chicken in Subang area were not vaccinated of ND since 2004

Table 4. The result of mean and distribution antibody titre of vaccinated broiler in Subang area using geometric mean tire (GMT) and coefficient of variation (CV)

Subdistrict	Type of chicken	Age	∑ Population (individual)	% *Pool*+ M		∑ Tested serum	Range Antibody titre	GMT	CV
				C	O		(Log2)	(Log2)	%
Binong	Layer	40 mg	120	0	0	15	0	0	0
Binong	Broiler	2 mg	10.000	80	20	35	0	0	0
Binong	Broiler	3 mg	20.000	75	87	50	0–7	0.8	45.3
Cipunagara	Broiler	2mg	18.000	0	0	8	0	0	0
Cipunagara	Broiler PS	culled	14.150	64	27	30	0–5	1.1	185
Cipunagara	Broiler PS	culled	950	40	70	64	0–8	2.8	49.9
Total						212			

CV ≤35% = sebaran of homogenous titre; CV >35% = sebaran of not homogenous titre

it, where the flock with low antibody titre is protective against NDV, in field was still occur often (Yan et al. 2011). Besides, according to Reynolds & Maraga (2000) and Erf (2004) experimentally showed there was no directly correlation between antibody titre in serums against chicken immunity which was challenged with NDV.

Antibody titre response is strongly influenced by quality of vaccine, route and the implementation of application, the environment, individual's factor and the species of bird (OIE 2012). Massal vaccination using live vaccine is often used than individually vaccine, because it is cheaper and easier to be applied (Senne et al. 2004). Vaccine from virulent strain (LaSota and B1) is commonly used around the world to overcome ND because it can provide protection to virulent NDV if the vaccination is done correctly (Kapczynski & King 2005; Cornax et al. 2012; Dortmans et al. 2012). In reality, sometimes the results are not as expected, mass vaccination using spray method showed that the percentage of group immunity was 53%, whereas vaccination by drinking water showed that the percentage of group immunity was 60% (Degefa et al. 2004). Ineffective vaccination also may be affected by immunosuppressive conditions of birds (Perozo et al. 2012). Beside live vaccine, inactive vaccine is also often used in layer and breeder farming because it may provide longer high titre than the live vaccine and the antibody can be downgraded to their generation (Al-Garib et al. 2003). But inactive vaccine is expensive and its application must be done individually, so not all breeders can use it. Alike serology survey in vaccinated broiler and layer chicken in Faisalabad was also done by Numan et al. (2005), the result showed that antibody titre was varies in two types of the bird, but the majority antibody titre was ≥3 Log2. Aziz & Ahmed (2010) did

the serology survey of unvaccinated domestic chicken in Sulaiman Province, Irak. The result showed that serum of 500 tested chickens, 172 serums (34%) was detected antibody. This shows that ND still endemic in Irak.

The existence of a positive antibody specific to NDV in unvaccinated duck and native chicken serums showed that in Subang was vulnerable of ND. This is because the duck and chicken were positive for antibody against NDV may be a carrier which became a source of Newcastle disease spreading (Saepulloh & Darminto 2005; Adi et al. 2010).

Observing the presence of antibodies in 16 birds that excreting NDV from 9 sub districts showed that only 4 individual from 3 sub districts were positive with varying titres (Table 5). The antibody was detected in unvaccinated duck and did not show symptoms of illness from Sukasari area with antibody titre 2 Log2 (isolate 3), two weeks old vaccinated broiler chicken with illness symptom from Binong area with titre 3 Log2 (isolate 7), three weeks old vaccinated broiler chicken with illness symptom with titre 8 Log2 (isolate 8) and 12 weeks old vaccinated breeding broiler (parent stock) and showed illness symptom with titre 5 Log2 from Cipunagara (isolate 13) (Table 5). The existence of antibody in unvaccinated duck and was successfully isolated of NDV showed an immune response of ongoing NDV infection, while the detection of antibody in unvaccinated chickens and ducks where NDV was not found, according Rezaeianzadeh et al. (2011), it showed that NDV infection that has passed and the birds were able to survive and recovery from NDV infection. In the unvaccinated birds where NDV was not detected, according to Daulay (2005) who was successfully isolated NDV from 2 wild birds (pigeon and turtle doves) showed antibody titre 0 Log2 was due to infection process has not been occurred, so the antibody has not been produced. In 3 vaccinated domestic chickens and detected of antibody against NDV, showed high titre antibody in the range 3-8 Log2 may still excrete virus through the cloaca and oropharynx. This may be affected by ineffective vaccination due to vaccine virus with field NDV was not homologous, so virus shedding occurred. Antibody response was not formed due to suboptimal vaccination, so that the chickens may be infected by NDV according to no detected antibody of vaccinated domestic chickens and isolated of NDV. The result of testing sample serum by HI test which successfully isolated from NDV may be seen in Table 4.

Antigenic diversity

Homologous antiserum will has an higher affinity with the viral surface epitopes so its more optimal inhibiting hemagglutination activity. Characterization of 18 antigen isolates with the HI test using antisera Lasota (lentogenic) obtained the average (mean) of HI titres between 6-8 log2 with antisera and Komarov (mesogenic) between 9-13 log2. Isolates affinity with LaSota antisera showed relatively homogeneous antigenic character, only a few isolates that showed variation reaches 2 log2 (1st, 2nd, and 3rd isolate), as well as antisera Komarov, only a few isolates that showed variation reaches 4 log2 (3rd, 6th, and 16th isolate). All NDV isolates showed a higher affinity against antisera Komarov compared with Lasota antisera that indicates all the NDV tend to get into a virulent NDV strain (Figure 2). According to Alexander & Senne (2008) differences of antigenic between NDV strains which may be recognized by specific antibodies determined by a hemagglutinin (HN) protein. Beside it, according Adu (1985) and Ibu et al. (2008) antigenic variation in NDV from the same strainoccurs because of various functions of the external proteins due to mutation.

Study of the antigenic diversity of NDV using polyclonal antisera had been conducted by Emilia (2013) using antisera strain Lasota, Komarov, the G7 and the ITA against four isolates Serpong area, West Java. The result showed that varies affinity with HI titres ranging between 3-5 log2 (Lasota), 5-10 log2 (Komarov), 6-8 log2 (G7) and 3-7 log2 (ITA). Hsiang-Jung et al. (2004) examined the variation of antigenic against 36 isolates from Taiwan NDV obtained between 1969-1996 using 22 monoclonal antibodies (MAB) and was able to separate the 36 isolates into 18 groups antigenic and based on the nucleotide sequences of gene F were grouped into 15 genotypes. Characterization of NDV antigen may also be done with a monoclonal antibody (MBA). Hu et al. (2010) showed using four types of MBA, that NDV antigenic variation may occur due to mutations of residues K (Lysine) at position 347 in the HN protein.

Characterization of physical properties of ND Virus with Elution Time Test

Determination of NDV strain may be known by its biological activity, including by elution time test. Significantly different in the time value may be used to distinguish of NDV strain on field roughly. According to Ezeibe & Ndip (2005) elution time of velogenic patotype virus had elution time between 84-189 minute, while mesogenic virus had elution time between 45-78 minute and virus that includes lentogenic (LaSota) had elution time for 20-43 minutes. Characterization of the pathogenicity of 18 isolates with elution showed 3 isolated include mesogenic group and 15 isolates to velogenic group (Table 5). Observing the elution time of isolates obtained from cloacal and oropharyngeal swabs from 1 native chicken in Compreng area (isolate

Table 5. Result of detection of specific antibody against NDV with HI test in 16 bird's serums which successfully isolated of NDV in Subang area

District	Type of bird	%	Antibody	Isolate	
		Pool+ M	Titre(Log2)	Code	Type of sample
Ciasem	Duck	40%	0	1	C
Pusaka Negara	Duck	40%	0	2	C
Sukasari	Duck	80%	2	3	C
Tambak Dahan	Kp. chicken	42%	0	4	C
Tambak Dahan	Kp. chicken	42%	0	5	C
Binong	Broiler	69%	0	6	O
Binong	Broiler	69%	3	7	O
Binong	Broiler	69%	8	8	O
Binong	Broiler	69%	0	9	O
Compreng	Kp. chicken	50%	0	10a	C
Compreng	Kp. chicken.	25%	0	10b	O
Pagaden	Kp. chicken	67%	0	11	C
Pagaden	Kp. chicken	66%	0	12a	C
Pagaden	Kp. chicken	67%	0	12b	O
Cipunagara	Broiler Ps.	31%	5	13	O
Cipunagara	Broiler Ps.	31%	0	14	O
Subang	Kp. chicken	83%	0	15	C
Subang	Kp. chicken	83%	0	16	C

Kp= kampong; Ps= parent stock; C= cloaca; O= oropharynx

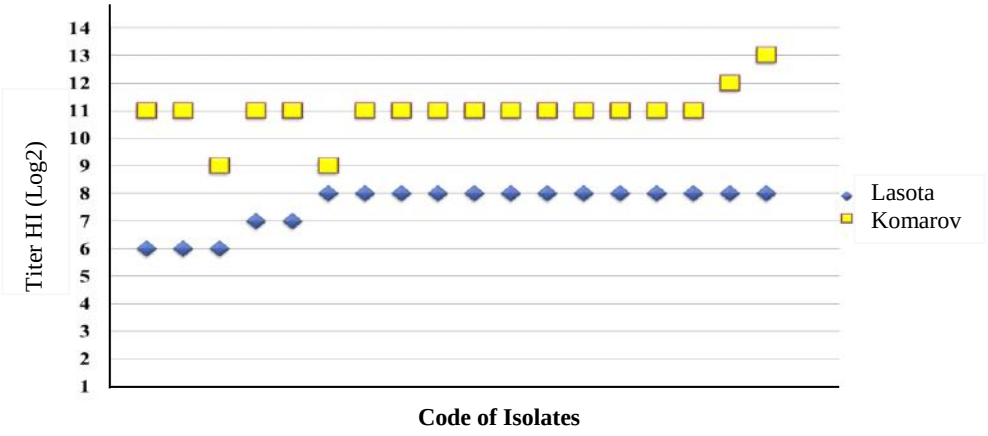


Figure 2. Comparison of antigenic of 18 isolates with HI test. The majority of HI titres looks homogeneous, only a few isolates that showed a variation with the antisera Lasota 2 log2 and with antisera Komarov 4 log2. HI titre comparison of both antisera showed isolates tend to lead to a virulent strain

Table 6. The result of elusion time test of NDV isolates that obtained from chicken and duck in 10 areas in Subang area

District	Type of Bird	Code of isolate	Type of samples	Elusion time (minute)	Type of Patotipe
Ciasem	Duck	1	C	117±0.0	Velogenic
Pusaka Negara	Duck	2	C	119±0.0	Velogenic
Sukasari	Duck	3	C	162±0.0	Velogenic
Tambak Dahan	Kp. Chicken	4	C	165±5.2	Velogenic
Tambak Dahan	Kp. Chicken	5	C	117±0.0	Velogenic
Binong	Broiler	6	O	89.6±23.7	Velogenic
Binong	Broiler	7	O	225±0.0	Velogenic
Binong	Broiler	8	O	232±12.1	Velogenic
Binong	Broiler	9	O	105±0.0	Velogenic
Compreng	Kp. Chicken	10a	C	219±0.0	Velogenic
Compreng	Kp. Chicken	10b	O	262.3±0.0	Velogenic
Pagaden	Kp. Chicken	11	C	117±0.0	Velogenic
Pagaden	Kp. Chicken	12a	C	80±0.0	Mesogenic
Pagaden	Kp. Chicken	12b	O	70±0.0	Mesogenic
Cipunagara	Kp. Chicken	13	O	92.6±21.5	Velogenic
Cipunagara	Kp. Chicken	14	O	76.3±1.2	Mesogenic
Subang	Kp. Chicken	15	C	117±0.0	Velogenic
Subang	Kp. Chicken	16	C	162±0.0	Velogenic

Kp= kampong, 10a and 10b; 12a and 12b= from the same individual

10a and 10b) and isolates obtained from cloacal and oropharyng swabs from 1 native chicken in Pagaden area (isolate 12a and 12b) were included to the same strain that was mesogenic/mesogenic and velogenic/velogenic, and HI titre from each isolate also had the same value. It indicated that both of excreted isolates from cloacal and oropharinx from 1 individual were the same NDV, but to confirm the virus identity, it needs to be sequenced. Elution time may be affected by temperature. According to Hussain et al. (2008) the elution time will be longer at 4°C and will be faster when the temperature is raised, therefore in order to obtain an accurate results, the elution test should be performed at a steady temperature. Besides, the elution test was also influenced by the concentration of red blood cells, if there are too many red blood cells during the testing, the red blood cells are not able to be bound by the virus, so that looks like a reaction to release of red blood cells by the virus and this will reduce the efficiency of elution test (Ezeibe & Ndip 2004).

Characterization of ND Virus by rRT-PCR fusion (F)

Characterization of 18 isolates by rRT-PCR fusion (F) showed positive result for all isolates, indicating that all isolates were virulent virus strain. The result of rRT-PCR F test showed there was a correlation with the result of elusion time test where was obtained mesogenic isolates and 15 velogenic isolates, this is correspond with the result of rRT-PCR F that showed all isolates were positive for virulent NDV strain. Not all virulent NDV may be detected by rRT-PCR using primer fusion (F). According to Kim et al. (2006) specificity in molecular diagnostic tests, may be affected by nonconformities between nucleotide pairs of primer base and probe with sequence that have a potential to give a fake negative result, while according to Cattoli et al. (2011) high degree of nucleotide variation in gen F may cause incompatibility between the primers and probes with the NDV amino acid

sequence. The incompatibility of primer oligobucleotide cause hybridization between primer/probe with RNA of virus not occurs, so it was not detected by real-time PCR system software.

CONCLUSION

Newcastle disease was still endemic in Subang area and the infection may be subclinical. Eighteen ND viruses which were found, majority had relatively homogeneous character, just a few isolates that showed diversity of pathogenicity and antigenicity (antisera LaSota: isolate 1, 2, and 3; antisera Komarov: isolate 3, 6 and 16). The ducks from Cipeundeuy area had not been exposed of NDV. The distribution of antibody titres in vaccinated chicken was unequal. The result of this study contributed information about ND in Subang area and may be used as feedback for Subang government to determine the prevention and control programs of ND infection in poultry in Subang.

RECOMMENDATION

Further research needs to be done to see the acid-base sequences of cleavage site of fusion (f) protein by sequencing or *pathotyping* test (MDT, ICPI, and IVPI) in order to obtain more information about pathogenicity and NDV strains which spread in Subang area. Sequencing needs to be done to see mutation in hemaglutinin. Strict sanitary and vaccinate use a combination of homologous live and inactive vaccine with field NDV can be applied to prevent ND infection in birds in Subang. Besides, more extensive surveys with more samples needs to be done to get information about infection of NDV that cover all areas in Subang area.

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Curcumin Effect on Bleomycin-Induced Pulmonary Fibrosis in *Mus musculus*

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ABSTRAK

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Kurkumin merupakan bahan aktif utama dari tanaman kunyit (*Curcuma longa*) diketahui memiliki aktivitas sebagai anti-oksidan dan anti-inflamasi. Bleomisin merupakan salah satu obat anti-kanker yang dapat menginduksi fibrosis paru-paru pada manusia dan hewan. Tujuan penelitian ini adalah untuk mengetahui efek biologis kurkumin pada fibrosis paru-paru yang diinduksi bleomisin pada mencit. Sebanyak 16 ekor mencit galur ddy dibagi dalam 4 kelompok perlakuan: (i) kontrol, 100 µl aquadest steril diinjeksikan secara SC, (ii) bleomisin (BLM), 100 µl bleomisin konsentrasi 1 mg/ml diinjeksikan secara SC, (iii) kurkumin (CMN), 100 µl aquadest steril diinjeksikan secara SC dan 100 mg/kg BB kurkumin dalam 0,5% carboxymethylcellulose (CMC) yang diinjeksikan secara IP, dan (iv) BLM+CMN, 100 µl bleomisin dengan konsentrasi 1 mg/ml diinjeksikan secara SC dan 100 mg/kg BB kurkumin dalam 0,5% CMC diinjeksikan secara IP. Semua perlakuan diberikan setiap hari selama 4 minggu. Organ paru-paru dikoleksi dalam 10% buffered neutral formalin (BNF). Pengamatan histopatogi dengan pewarnaan hematoksilin-eosin (HE) dan Masson's trichrome (MT) untuk diamati tebal dinding alveol dan luas daerah jaringan ikat. Hasil penelitian menunjukkan bahwa pada kelompok perlakuan bleomisin (BLM) terjadi peningkatan luas jaringan ikat dan tebal dinding alveol secara signifikan jika dibandingkan dengan kontrol. Sementara itu, pemberian kurkumin pada mencit yang mendapatkan induksi bleomisin (kelompok BLM+CMN), menyebabkan terjadinya penurunan signifikan luas jaringan ikat dan tebal dinding alveol. Dapat disimpulkan kurkumin memiliki aktivitas sebagai inhibitor fibrogenesis untuk mengurangi keparahan fibrosis paru-paru akibat aplikasi bleomisin pada mencit.

Kata Kunci: Bleomisin, Fibrosis Paru-paru, Mencit, Kurkumin

ABSTRACT

Rahmi A, Setiyono A, Juniantito V. 2015. Curcumin effect on Bleomycin-induced pulmonary fibrosis in *Mus musculus*. JITV 20(2): 148-157. DOI: http://dx.doi.org/10.14334/jitv.v20i2.1169

Curcumin, a curcuminoid compound of turmeric has been demonstrated to have anti-oxidant and anti-inflammatory properties. Bleomycin (BLM) is an anti-cancer drug induced pulmonary fibrosis in human and animals. This study was aimed to investigate biological effects of curcumin on bleomycin-induced pulmonary fibrosis in mice (*Mus musculus*) through pathomorphological assessment. In this study, 16 mice ddy strain were divided into four groups, namely (i) control, mice were subcutaneously (SC) injected with 100 µl sterilized aquadest in dorsal skin, (ii) BLM group, injected SC with 100 µl of 1 mg/ml BLM in dorsal skin, (iii) Curcumin (CMN) group, mice were intraperitoneally (IP) injected with 100 mg/kg body weight (BW) curcumin dissolved in 0,5% carboxy methyl cellulose (CMC) and injected with 100 µl sterilized aquadest SC, (iv) BLM+CMN group, injected SC with 100 µl of BLM 1 mg/ml and injected IP with 100 mg/kg BW CMN in 0,5% CMC. All treatments were performed daily for four weeks period. The lung samples were collected and fixed in buffered neutral formalin (BNF) 10%. Histopathological evaluation was performed with hematoxylin-eosin (HE) and Masson's trichrome (MT) stains. The results showed that BLM treatment significantly increased fibrosis area and alveolar wall area fraction as compared to control. OIn the other hand, CMN treatment significantly reduced fibrosis area and alveolar wall area fraction in mice treated with BLM. In conclusion, our study showed that CMN treatment may inhibit lung fibrogenesis in BLM-induced pulmonary fibrosis.

Key Words: Bleomycin, Curcumin, Mice, Pulmonary Fibrosis

INTRODUCTION

Pulmonary fibrosis is a chronic pulmonary disease characterized by pathological lesions in the form of extracellular matrix and tissues component changes, followed by clinical symptoms, physiological disorders, and radiographic findings (Todd et al. 2012). Many factors cause this pulmonary fibrosis, such as it is exposured by air poison, particular pulmonary disease, effect of radiation therapy and chemotherapy (Ley & Collard 2013).

Bleomycin (BLM) is used as a chemotherapy agent for various cancers. This antibiotic of antitumor is a derivative glycopeptide isolated from *Streptomyces verticillus* (Yamamoto 2010). BLM has strong antitumor activity. Clinically, BLM is used as tumor therapy such as *squamous cell carcinoma* at around head and neck (including mouth, tongue, tonsils, nasopharynx, oropharynx, sinus, palate, lips, buccal mucosa, the gums, the epiglottis, and larynx), *malignant lymphoma, testicular carcinoma,* and *malignant pleural effusion* (Chu et al. 2010).

The main limitation of BLM therapy is its side effect which causes alveolitis fibrosis (Matsushita et al. 2008). Frusch et al. (2012) said that there are several syndromes in the lung linked with BLM utilization, that is bronchiolitis obliterans, hypersensitivity eosinophils, and interstitial pneumonitis which will thrive become pulmonary fibrosis. Besides, BLM-induced pneumonitis can reach 46% in patients who receive BLM treatment. Mortality level of the pulmonary fibrosis disease is around 10-20% with 2-3% from patients treated by BLM. Brugge et al. (2013) said that BLM therapy side effect could cause pneumonitis.

Fibrosis mechanism in lung due to the side effect of BLM utilization has not known. Some factors that have role in pulmonary fibrosis in BLM therapy are oxidative stress, BLM-hydrolase enzyme deactivation, genetic, and the release of inflammatory cytokines (Brugge et al. 2013; Reinert et al. 2013). BLM use in human also causes other side effect, such as an abnormality skin characterizes the scleroderma (Juniantito et al. 2013).

Fibrosis pathogenesis (fibrogenesis) is divided in several overlapping phases, namely inflammation, connective tissue proliferation, and intractable fibrosis (Matsushita et al. 2008; Reinert et al. 2013). Retardation in one step of fibrogenesis causes a decreasing of connective tissue formation (Loomis-King et al. 2013). BLM could stimulate endothelial cells, macrophages, and fibroblasts to induce synthesis of inflammatory mediators especially proinflammatory and fibrogenic cytokines, inducing apoptosis, and free radicals synthesis (Yamamoto 2010; Yamamoto & Katayana 2011). It was thought that prevention of fibrosis might be mediated by inhibition of inflammation with anti-inflammatory substances (Basnet & Skalko-Basnet 2011; Kardena & Winaya 2011).

Curcumin (diferuloylmethane) is an active ingredient in turmeric, in addition, it is also known as an antioxidant (Zhang et al. 2011). Turmeric is widely available plant in Indonesia which is used often as spice and herbal ingredient. Anti inflammation effect of curcumin is likely because of a high pleiotropic molecule which able to interact with and related to the most transcription factors in inflammatory mediators synthesis and inhibiting free radicals releasing in the inflammatory cells (Jurenka 2009; Marçal et al. 2012).

Some studies have been conducted to determine curcumin effect as anti-inflammation and its potential to prevent the fibrosis (Jurenka 2009; Beevers & Huang 2011; Kardena & Winaya 2011). Curcumin can decrease fibrosis level in liver, kidney, and lung of laboratory animal (Beevers & Huang 2011). Therefore, curcumin can be used as potential candidate of anti-fibrosis preparate, especially in pulmonary fibrosis. This study was aimed to see the curcumin potential in preventing the side effect of BLM use in cancer patients who are undergoing a chemotherapy.

MATERIALS AND METHODS

Time and place

This study was conducted during December 2014 - March 2015 in Laboratory Animals Management Unit (UPHL), Faculty of Veterinary Medicine, Bogor Agricultural University and Histopathology Laboratory, Pathology Division, Faculty of Veterinary Medicine, Bogor Agricultural University.

Inducer material of pulmonary fibrosis

Bleocin® (Bleomycin hydrochloride 15 mg, Kalbe Farma, Jakarta, Indonesia) was diluted in 15 ml sterile aquadest to reach concentration of 1 mg/ml. As much as 100 µL of the solution was injected subcutaneously (SC) on the back skin for BLM and BLM+CMN group everyday for 4 weeks.

Curcumin

Curcumin active ingredients (Biopurify, Chengdu, China) was diluted in 0.5% *carboxymethylcellulose* (CMC) and injected 100 mg/kg of body weight by intraperitoneal injection (IP) in CMN and BLM+CMN group everyday for 4 weeks.

Experimental procedure

All of procedures conducted in this study met the requirement of Animal Ethics Commission of Bogor Agricultural University Number 25-2014 IPB. Sixteen 4 weeks old male ddy strain mice with body weight around 20-25 gram (The National Agency of Food and Drug Control (NA-DFC), Jakarta, Indonesia) were used and divided into 4 groups. There were 4 mice in each group.

This study was divided into 4 treatment groups, namely: (i) control, injected subcutaneously by 100 µL sterile aquadest in the back skin, (ii) BLM, injected

subcutaneously in the back skin with 100 µL BLM of 1 mg/mL concentration, (iii) CMN, injected subcutaneously with 100 µL sterile aquadest in the back skin and injected intraperitoneally with curcumin of 100 mg/kg of body weight in 0.5% CMC, and (iv) BLM+CMN, injected subcutaneously with 100 µL BLM of1 mg/mL concentration and injected intraperitoneally with curcumin of 100 mg/kg of body weight in 0.5% CMC. Injection of the curcumin by intraperitoneal injection was adapted from Li et al. (2013).

This study was done in three steps, namely: (i) acclimatization, the experimental animals were adapted in new cage for 2 weeks, (ii) treatments, the experimental animals were treated in accordance to the respective groups everyday for 4 weeks, (iii) termination, the experimental animal were euthanized by giving Ketamine HCL of 0.2 mL/head (AVMA 2013). Furthermore, left lobus of lung was collected to be made histopathology preparates, then stained by hematoxylin-eosin (HE) for observation of tissue structure of the lung (Fischer et al. 2006) and Masson's*trichrome* (MT) to see the presence of connective tissue (Suvik & Effendy 2012).

Histopathological assesment by HE and MT stains

Lung of BNF 10% was sliced in 3 mm and inserted into *tissue cassette* for dehydration process, clearing and paraffin infiltration using automatic tissue processor. Chunk organs, further was printed in the paraffin until paraffin block formed. For histology test, the paraffin block was sliced by rotary microtome in 3-5 µm of thickness. Cutting results were placed in object glass to be deparafinized and rehydrated for tissue staining by hematoxylin-eosin and Masson's *trichrome*. All of the cutting results were observed by a light microscope which was connected to the computer. Observed parameters of this organ were connective tissue width and alveoli wall thickness.

Connective tissue width

Connective tissue width was quantitatively counted by *Image J®* software (http://imagej.nih.gov//ij/; NIH, Maryland, USA), by analyzing tissue slide which has stained by modification of MT stains (Suvik & Effendy 2012). *Image J®* software utilization is to decrease the level of counting subjectivity by scoring method. By using 40x objective lens magnification, as much as 20 visual fields per treatment group randomly selected by video camera (Indomikro® HDMI camera) which was shown on colored screen. The width of each visual field was 326.40×184.00 µm^2. Furthermore, figure was customized for contrast, brightness, and threshold color. Image analysis program detected the width of blue area which indicates collagen area or connective tissue in each visual field and presented in the form of percentage.

Alveolar wall thickness

Alveoli wall thickness quantitatively was counted by analyzing tissue slide which has been stained by HE. As much as 20 visual fields per treatment group were randomly selected using video camera showed on the colored screen. The wide of each visual field was 326.40×184.00 µm^2. Alveoli wall thick measurement was done 10 times of every visual field, and the data presented in the form of average.

Data analysis

Percentage of width of connective tissue and alveoli wall thickness were analyzed by SAS® 1.9 software for *Microsoft®Windows®* ANOVA and presented in form of average and standard deviation. Further, Duncan advance test was done to determine whether there is a significant differences between the treatments or not.

RESULT AND DISCUSSION

Histology Result by Hematoxylin-Eosin Staining

Figure 1 is photomicrography of lung tissue of each treatment. Figure 1A is a photomicrography of control group of lung tissue. In this figure, normal alveoli wall structure is 3.7±0.6 µm. The same thing occurred in CMN group (Figure 1C). It is shown that lung with normal alveoli wall structure of 3.4±0.5 µm.

Figur 1B is a photomicrography of BLM group. In this group, it is shown an inflammation accompanied by an increase in cell number in interstitium, therefore alveoli wall become thicker (24.9±6.3 µm) than the control group. Besides, there was an infiltration of inflammatory cells dominated by macrophages and lymphocytes. Alveoli wall thickening resulted in width of alveoli area became smaller, therefore the air entered the lung became limited. Rienert et al. (2013) said that thickening of the alveoli wall would cause difficulty in breathing (anoxia). In the BLM+CMN group (Figure 1D), it is shown lesion similar to the BLM group, that is the presence of inflammation, increasing in cell number in interstitial and the thickening of the alveoli wall (9.9±2.6 µm) with lower severity compared to the BLM group.

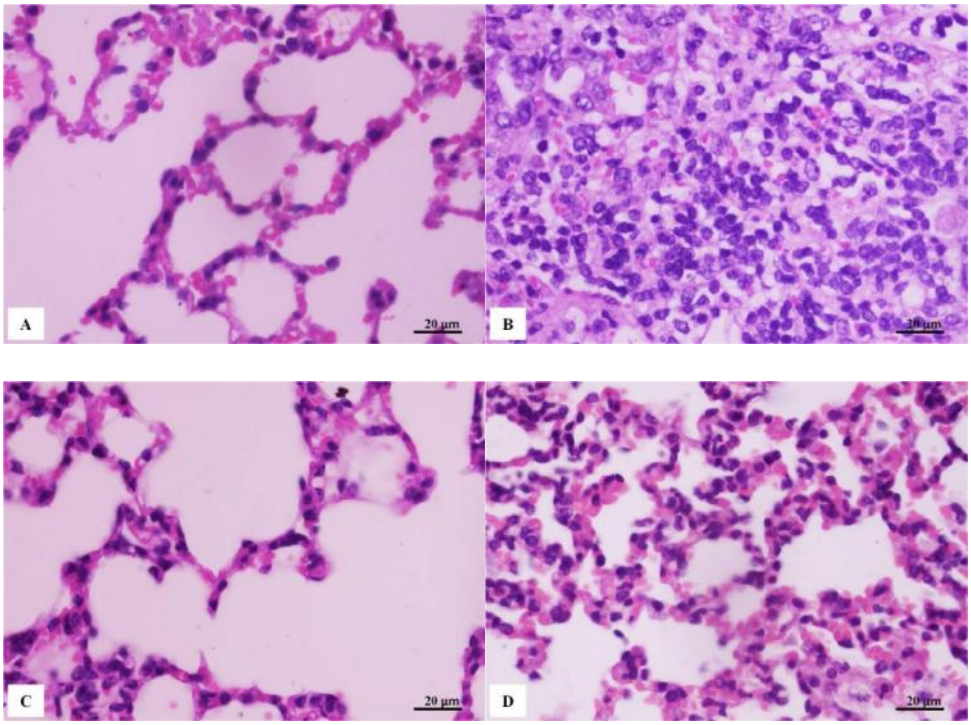

Figure 1. Photomicrography of lung tissues by HE staining of each treatment group: Control (A); BLM (B); CMN (C); and BLM+CMN (D)

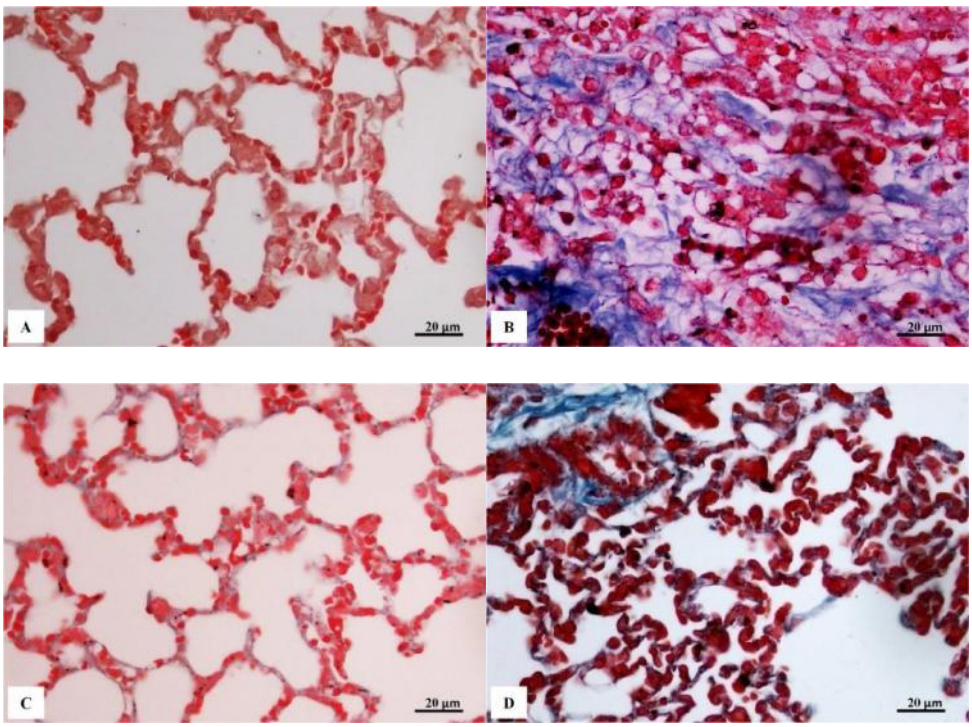

Figure 2. Photomicrography of lung tissues by MT staining of each treatment group: Control (A); BLM (B); CMN (C); BLM+CMN (D)

Yamamoto & Nishioka (2000) said that in mice everyday induced by BLM SC for 4 weeks, there was pulmonary fibrosis characterized by mononuclear cell infiltration, ederma, and septum alveolar thickening due to decrease in number of macrophages alveolar and lymphocyte. Besides, Izbicki et al. (2002) found that there were perivascular and lymphocytes peribronkial (day 3), subcapsular fibrosis (day 6), and decreasing in number of macrophages alveolar and septum alveolar (day 14) in mice's lung histology induced by pulmonary fibrosis with single dose BLM by intratracheal.

Histology result by Masson's Trichrome (MT) staining

Tissue staining techniques by MT is addressed to identify the presence of the connective tissue or collagen in the tissue. Result of the lung tissue staining by MT technique was presented in Figure 2.

Figure 2A (control) and 2C (CMN) shows normal structure of lung tissues with thin alveoli wall (3.7±0.6 μm). In the two figures do not show an excessive blue structure, indicating that in the two group there is no excessive collagen accumulation. On the contrary, figure 2B (BLM) shows blue accumulation which extends almost to the entire visual field. The blue color on lung slide colored by MT indicating the presence of accumulation connective tissue or collagen (Suvik & Effendy 2013). Besides, in the BLM group was also seen smaller alveoli diameter compared to the control group. Figure 2D (BLM+CMN) shows light accumulation of collagen visualized by blue color and looked like thickening of the alveoli wall.

Width of connective tissue

Measurement of connective tissue width in the lung was done to determine extent of lung tissue damage due to BLM application. In figure 3, it is not seen the presence of a significant differences of the connective tissue between control group (2.4±0.8%) and CMN group (1.5±0.6%). However, in BLM group (15.0±2.4%), there was significantly wider connective tissue value ($P<0.05$) compared to control group. In the BLM+CMN group (6.2±2.0%), there was significantly lesser width connective tissue ($P<0.05$) compared to the BLM group. This research result was in line with Izbicki et al. (2002) that there was an increase in connective tissue width of mice lung induced by single dose Bleomycin by intratracheal. Besides, Liu et al. (2013) also reported that there were an inflammation and fibrosis in interstitial of mice lung tissue induced by BLM through intratracheal.

Wide development of connective tissue in BLM group was caused by BLM activity which may induce pulmonary fibrosis by causing inflammation, than it developed become proliferation of fibroblast. Fibroblast is a cell which produced extracellular matrix (Todd et al. 2012). Yamamoto & Katayama (2011) said that BLM can stimulate endothelia cell, macrophage, and fibroblast to induce inflammatory mediator. The inflammatory mediator will induce proinflammatory cytokines, cytokine fibrogenic, transforming growth factor-β (TGF-β), apoptosis, and the release of free radicals.

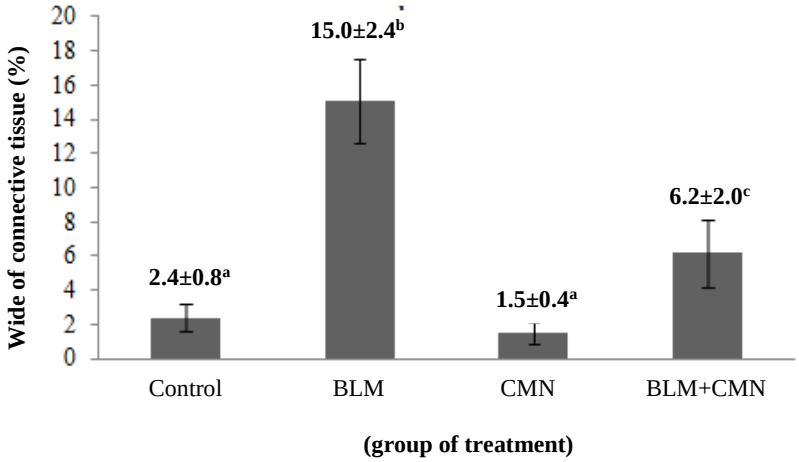

Figure 3. The wide of connective tissue of each treatment group, different superscript word shows significant difference ($P<0.05$)

Reinert et al. (2013) reported that low *BLM-hydrolase* enzyme activity can be a predisposing factor of pulmonary fibrosis due to the BLM application. Through in vivo, the BLM will be metabolized become non-toxic molecule with *Bleomycin-hydrolase* enzyme help (Haston et al. 2002). This enzyme is cytosolic aminopeptidase group which has low activity in lung and skin (Reinert et al. 2013). Therefore, BLM application may cause the pulmonary fibrosis. The fibrosis in the lung is started by an inflammatory. The inflammatory will develop into proliferation of fibroblasts into myofibroblast (cell produces collagen), and it finished by persistent formation of connective tissue. Mechanism of fibrosis induction by BLM was caused by increasing secretion of extracellular matrix proteins because of cytokine fibrogenic effect which was released by macrophages, endothelial cells, and fibroblasts (Reinert et al. 2013). Besides, BLM in the cells will bind with iron ion (Fe^{+2}) and oxygen forming Fe^{+3} ion which produce the free radicals, so that cause DNA damage, RNA degradation, and inducing the apoptosis (Yamamoto 2010). Moreover, the free radicals or reactive oxygen species can produce direct toxicity through participation in redox reactions and subsequent fatty acid oxidation, which leads to membrane instability. Oxidant can cause inflammatory reaction within be lung. The inflammatory mediators like cytokines such as interleukin-1, macrophage inflammatory protein-1, platelet-derived growth factor, and TGF-β are released from alveolar macrophages in animal models of bleomycin toxicity, resulting in fibrosis. Damage and activation of alveolar epithelial cells may result in the release of cytokines and growth factors that slimulate proliferation of myofibroblast and secretion of pathologic extracellular matrix, leading to fibrosis.

The inflammation due to BLM application will cause body to response to repair the tissue by involving connective tissue reconstitution from the fibroblast into myofibroblast (Reinert et al. 2012). Myofibroblast is the main component in fibrosis process or wound healing. This myofibriblast will produce extracellular matrix, so it will cause extracellular matrix proteins accumulation. Previous study conducted by Xu et al. (2009) showed that TGF-β is cytokine inducer of differentiation of fibroblast cell into myofibroblast cell. Yamamoto & Nishioka (2004) presented that several actions of TGF-β are, among other as chemotactic macrophages and fibroblasts, stimulating fibroblast proliferation, increasing extracellular matrix synthesis, decreasing extracellular matrix proteinase regulation, and increasing proteinase inhibitor regulation. So that exacerbates the development of fibrotic lesions in the lung.

The opposite occurred in BLM+CMN group where there was a significant decrease ($P<0.05$) of width of connective tissue compared to the BLM group (Figure 3). In line with Punithavathi et al. (2000) that there was a decreasing of alveolar macrophage amount, superoxide and nitrikoksida in rats given oral curcumin treatment after single dose Bleomycin induction by intratracheal. So, in his research, it was concluded that curcumin was a potential material as an anti-inflammatory and anti-fibrogenic in BLM-induced pulmonary fibrosis.

Bleomycin as an antibiotic antitumor agent, widely used for livestocks, such as pig (Balazs et al. 1994) and sheep (Organ et al. 2015). Same as in laboratory animals, BLM utilization in pig and sheep also caused side effect such as an inflammation into pulmonary fibrosis (Balazs et al. 1994; Organ et al. 2015). Pathogenesis mechanism of pulmonary fibrosis due to BLM application in livestocks was alike with the laboratory animals, so that was possible to be given curcumin as anti-inflammation and antioxidant to reduce fibrogenesis severity in the pulmonary fibrosis cases due to BLM application.

The decreasing of width of lung connective tissue in this study may be caused by 2 factors. First, the curcumin can directly inactivate the bleocymin activity, so that inflammation did not occur. Second, activity of anti-inflammation from the curcumin causes the inflammation become lower, so that fibrosis can be pressed. The second one is closer with pathogenesis of fibrosis inhibition by the curcumin. This was caused by BLM induction and curcumin therapy conducted through 2 different ways. Bleomycin induction was done subcutaneously, whereas curcumin therapy was done by intraperitoneal. Both of subcutaneous and intraperitoneal ways will interact systematically in the body, so that increasing width of lung connective tissue due to Bleomycin application possibly hampered by anti-inflammation activity from the curcumin. Besides, the curcumin was known to have effectiveness as an inhibitor receptor TGF-β, so that TGF-β production can be inhibited (Mo et al. 2012).

Prasad et al. (2014) said intraperitonel injection of curcumin more often applied to animals than to human. Besides, the bioavailability compound of curcumin via intraperitoneal injection is higher than gavage. Interestingly, oral curcumin treatment showed no effect on important measure of BLM-induced injury in mice, whereas intraperitoneal curcumin administration effectively inhibited inflammation and collagen deposition along with a trend toward improved survival of animal, and also reduced fibrotic progression even when administered after the acute bleomycin-induced inflammation had subsided (Smith et al. 2010).

Smith et al (2010) said that curcumin administration could significantly prevent lung inflammation and collagen deposition in pulmonary fibrosis induced by BLM by intratracheal. Curcumin was high pleiotropic

molecule which able to interact and bind with the most of inflammation molecule target (Jurenka 2009; Marçal et al. 2012). Curcumin has been reported having activity as an anti-cancer (Wiken et al. 2011), anti-oxidant (Bhullar et al. 2013) and anti-inflammation (Basnet & Skalko-Basnet 2011). Turmeric bioactive compounds had protective effect to heart cell which exposed to toxic chemicals such as tetrachloride (CCl_4) (Prakash et al. 2008; Kardena & Winaya 2011). Yu et al. (2011) reported that curcumin administration can reduce pancreatic tissue damage and another organs due to *caerulein* induction through inhibiting the release of inflammatory mediator of TNF-α cytokines.

Curcumin has been reported to exhibit anti-tumorigenic and chemo-preventive activities due to the structural resemblance of dibenzoyilmethane (DBM) to the anti-inflammatory and aspirin-like skeleton of DBM derivates (Lin et al. 2011). Villegas et al. (2011) reported curcumin has also demonstrated protective and preventive effect in progression of colorectal colitis cancer, which was collerated with a lowered immune-reactivity of beta-catenin and reduction of pro-inflammatory cytokine levels and a decrease of inflammatory mediator's overexpression.

Lee et al. (2010) evaluated that curcumin as a potential dietary supplement in the setting of thoracic radiotherapy in mice with creating a baseline radioprotective state prior to irradiation by inducing protective gene expression as well as having potent direct antioxidant scavenging activity. Li et al. (2013) said curcumin application has a protective effect on the acute hepatic injury induced by acetaminophen with reduced acetaminophen-induced hepatocyte apoptosis and that protection may be related to its inhibition of lipid peroxidation and oxidative stress.

Thickness of alveolar wall

Figure 4 shows that there is no significant difference of alveolar wall between control group (3.7±0.6 µm) and CMN group (3.4±0.5 µm). However, there was significantly (P<0.05) higher alveolar wall thickness value in BLM group (24.9±6.3 µm) compared to the control group. In BLM+CMN group (9.9±2.6 µm) there was significantly (P<0.05) lower alveolar wall thickness value compared to BLM group.

The results about width of connective tissue and alveolar wall thickness in each treatment group were presented in Table 1.

This research result is aligned with a research of Izbicki et al. (2002) where alveolar wall thickening in mice lung tissue induced by single dose BLM by intratracheal. Alveolar wall thickening in BLM group

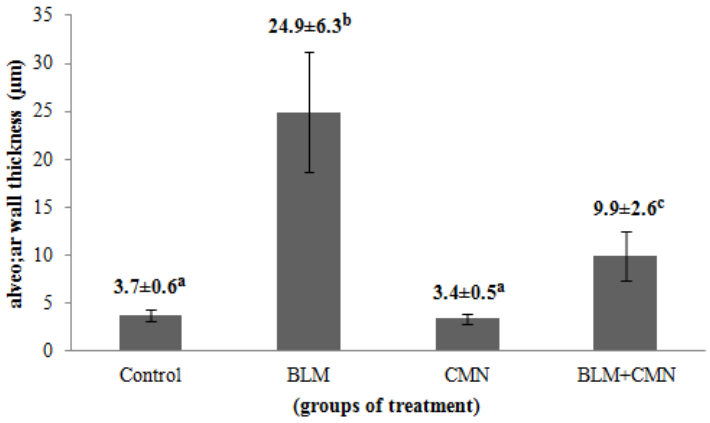

Figure 4. The Alveolar wall thickness in each treatment group, different superscript word shows significantly (P<0.05) difference

Table 1. Result of the wide of connective tissue and alveolar wall thickness

Treatment Group	Wide of connective tissue (%)	Alveolar wall thickness (µm)
Kontrol	2.39±0.78[a]	3.72±0.56[a]
BLM	15.03±2.45[b]	24.93±6.26[b]
CMN	1.50±0.61[a]	3.39±0.54[a]
BLM+CMN	6.17±2.01[c]	9.95±2.60[c]

Different *superscript* in one same column shows significantly difference (P<0.05) n= 4

was caused by BLM administration continuously every day, which eventually caused chronic type lesio forming. The BLM continuous administration caused formation of progressive pulmonary fibrosis lesion (Schwaiblmair et al. 2012; Reinert et al. 2013). Increase wall thickness was caused by the existence of the fibrosis, besides it can also caused by interstitial edema (Izbicki et al. 2002).

In this study, Bleomycin toxicity was caused by low activity of BLM-*hydrolase* enzyme in the lung causing the BLM not metabolized into intoxic molecule form. Reinert et al. (2013) said that low activity of BLM-*hydrolase* enzyme in the lung resulted in an excessive apoptosis. Besides, the BLM can also stimulate endothelial cell, macrophage, and fibroblast to induce inflammatory mediator, apoptosis, and free radicals (Yamamoto & Katayama 2011). The most common symptomps from bleomycin-induced hypersensitivity pneumonitis are exertional dispnea and non-productive cough, with progressive pneumonitis dyspnea at rest, tachypnea, and cyanosis may occur (Sleijfer 2001).

Bleomycin is one of xenobiotis which able to induce radical oxygen synthesis excessively such as superoxide, hydrogen peroxide, peroxynitrite and hydroxyl radicals which is the mean mediator in inflammatory process in the lung (Oury et al. 2001). The inflammation will cause inflammatory cells entering the tissue and releasing cytokine inflammation mediator (Reinert et al. 2013). Furthermore, the cytokine caused fibroblast proliferation into myofibroblast. This inflammation and fibroblast proliferation will cause an increase of alveolar wall thickness to the pulmonary fibrosis (Izbicki et al. 2002).

Curcumin administration significantly press alveolar wall thickness incidence. The curcumin can press apoptosis and inflammation incidence; this is related to its activity as anti-oxidant and anti-inflammation (Smith et al. 2010; Bhullar et al. 2013; Basnet & Skalko-Basnet 2011). Smith et al. (2010) presented that curcumin administration significantly press pulmonary inflammation and collagen deposition in the pulmonary fibrosis induced by BLM. Punithavathi et al. (2000) said that the curcumin mediated the reduction of total number of *broncho-association limfoid follicles* (BALF) cell by preventing inflammatory cell crossing endotel and epithelial basal membrane towards the inflammation location. This is because of the curcumin stabilized the endotel and epithelial basal membrane. Therefore, the curcumin activity as anti-oxidant and anti-inflammation can press alveolar wall thickness incidence in the lung due to BLM administration.

CONCLUSION

Curcumin administration can inhibit fibrogenesis in mice pulmonary fibrosis due to the BLM application. This information can be used as first information to prevent side effect of BLM utilization to cancer patients who has to undergo chemotherapy.

ACKNOWLEDGEMENT

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