ISSN 0853-7380
E-ISSN 2252-696x

Accredited by LIPI
Certificate Number: 655/AU3/P2MI-LIPI/07/2015

Jurnal Ilmu Ternak dan Veteriner

IJAVS *Indonesian Journal of Animal and Veterinary Sciences*

Volume 21
Number 1
March 2016

PUSAT PENELITIAN DAN PENGEMBANGAN PETERNAKAN
BADAN PENELITIAN DAN PENGEMBANGAN PERTANIAN
KEMENTERIAN PERTANIAN

JITV	Volume 21	Number 1	Page 1-72	Bogor, March 2016	ISSN 0853-7380

Jurnal Ilmu Ternak dan Veteriner

IJAVS *Indonesian Journal of Animal and Veterinary Sciences*

JITV	Volume 21	Number 1	Page 1-72	Bogor, March 2016	ISSN 0853-7380 E-ISSN 2252-696X

Published by:
Indonesian Center for Animal Research and Development
Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture

Collaborated with:
Indonesian Society of Animal Science

Secretariat of IJAVS:
Jalan Raya Padjajaran Kav. E. 59, Bogor 16128 - Indonesia
Telephone (0251) 8322185
Fax (0251) 8380588
E-mail: criansci@indo.net.id; jitvnak@yahoo.com
Website: http://medpub.litbang.pertanian.go.id/index.php/jitv

Indonesian Journal for Animal and Veterinary Sciences is published four times a year in March, June, September and December.

PREFACE

In this edition, volume 21 no. 1 March 2016, we proudly present articles from various disciplines such as Animal reproduction; feed and nutrition technology; forages technology; and veterinary technology. The articles published in this edition are: "Effectivity of BS4 enzyme complex on the performance of laying hens fed with different ingredients"; "*In Vitro* protein digestibility and fermentability of mulberry (*Morus alba*)-Leucaena foliage mixed feed"; "Anaerobic fermentation effectively reduces concentration of total tannins in *Chromolaenan odorata*"; "Follicular dynamic and repeatability of follicular wave development in Peranakan Ongole (PO) Cattle"; "Chitosan nanoparticle of hCG (Human Chorionic Gonadotrophin) hormone in increasing induction of dairy cattle ovulation"; "Phylogenetic tree of Kuantan cattle by DNA barcoding"; "Relationship of extender and packaging system an the length of preservation and the quality of chilled semen of Boer goat"; "Determination of production capacity of Circulated Primordial Germ Cells (Circulated-PGCs) of KUB chicken using lysis buffer Ammonium Chloride Potassium (ACK)" and "Potency of antigenic and serologic tests based on CNTKCQTP linear epitope on H5N1 haemagglutinin for Avian Influenza".

Hopefully these articles would offer any benefit to readers and the end-users of technological innovation, and attract interests from other authors to contribute in the future.

Chief Editor;

Bogor, March 2016

Complete paper may be accessed through:
http://medpub.litbang.pertanian.go.id/index.php/jitv or
http://peternakan.litbang.pertanian.go.id/index.php?option=com_content&view=article&id=3633&Itemid=119 or
through database CAB DIRECT (www.cabdirect.org) or
Indonesian Scientific Journal Database (isjd.pdii.lipi.go.id)

Jurnal Ilmu Ternak dan Veteriner

IJAVS *Indonesian Journal of Animal and Veterinary Sciences*

Volume 21, Number 1, March 2016 ISSN 0853-7380 E-ISSN 2252-696X

LIST OF CONTENT

Effectivity of BS4 Enzyme Complex on the Performance of Laying Hens Fed with Different Ingredients

Sinurat AP, Purwadaria T, Haryati T

[1]*Indonesian Research Institute for Animal Production, PO Box 221, Bogor 16002, Indonesia*
E-mail: ap_sinurat@litbang.pertanian.go.id

(received 06-01-2016; revised 21-03-2016; accepted 29-03-2016)

ABSTRAK

Sinurat AP, Purwadaria T, Haryati T. 2016. Pengujian efektifitas enzim BS4 terhadap performan ayam petelur yang diberi jenis bahan pakan yang berbeda. JITV 21(1): 1-8. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1389

Penelitian dilakukan untuk menguji efektifitas enzim BS4 yang dihasilkan dengan pembiakan *Eupenicilium javanicum* terhadap performan ayam petelur yang diberi jenis pakan yang berbeda. Tiga ransum dengan kandungan gizi yang sama (protein kasar, ME, lysine tercerna, methionine + cystine tercerna, tryptophan tercerna, Ca and P tersedia) disusun sesuai dengan kebutuhan ayam petelur. Ketiga ransum tersebut mengandung 3 bahan pakan utaman yang berbeda yaitu jagung dan bungkil kedelai (kontrol), dedak 30% atau bungkil inti sawit (BIS) 20%. Ketiga ransum kemudian ada yang ditambahkan enzim BS4 dan ada yang tidak. Setiap ransum diberikan kepada 24 ekor (6 ulangan @ 4 ekor) ayam Isa Brown mulai umur 19 hingga 37 minggu dan diamati performannya. Data dianalisa secara statistik dengan rancangan faktorial 2 (enzymes) X 3 (bahan pakan). Hasil menunjukkan bahwa penambahan multi enzim BS4 kedalam ransum ayam petelur tidak mempengaruhi perubahan bobot badan, konsumsi pakan, mortalitas, berat telur dan tebal kerabang telur. Suplementasi enzim BS4 kedalam ransum ayam petelur nyata meningkatkan produksi telur ($P<0.05$) dan memperbaiki FCR ($P<0.01$), pada ketiga jenis bahan pakan yang diuji (jagung, dedak dan BIS). Penambahan enzim BS4 juga nyata meningkatkan warna kuning telur pada ransum yang mengandung 20% BIS. Ayam yang diberi BIS 20% menghasilkan telur yang lebih banyak dibandingkan dengan yang diberi jagung atau 30% dedak. Namun, kerabang telur ayam yang diberi pakan 30% dedak lebih tebal dari ayam yang diberi jagung atau BIS. Disimpulkan bahwa penambahan multi enzim BS4 dalam ransum cukup efektip meningkatkan performan ayam petelur.

Kata Kunci: Enzim BS4, Bungkil Inti Sawit, Dedak, Produksi Telur, Kualitas Telur

ABSTRACT

Sinurat AP, Purwadaria T, Haryati T. 2016. Effectivity of BS4 enzyme complex on the performance of laying hens fed with different ingredients. JITV 21(1): 1-8. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1389

An experiment was carried out to evaluate the effectivity of an enzyme complex produced by Eupenicilium javanicum BS4 on the performance of laying hens fed with different feed ingredients. Three diets were formulated with similar nutrients (protein, ME, digestible amino acids, Ca and available P) value to meet the nutrient requirement of laying hens. Diets were formulated based on maize, palm kernel cake (PKC) or rice bran. The diets were either supplemented or not with BS4 enzyme complex and fed to Isa Brown pullet from 19 to 37 weeks old. Each diet was fed to 24 birds and the performances were recorded. Data were analysed statistically with 2 X 3 factorial design with six replicates. Results showed that supplementation of BS4 enzyme into laying hens diet did not influence body weight change, feed intake, mortalities, egg weight and egg shell thickness but significantly increased egg production ($P<0.05$) and the FCR ($P<0.01$) in any feedstuff tested. It also improved egg yolk color score on diet contained 20% PKC. Laying hens fed 20% PKC produced more eggs than those fed maize or rice bran. The egg shell of laying hens fed rice bran were thicker than those fed with maize or PKC. It is concluded that BS4 enzyme complex is effective when supplemented into laying hens diet.

Key Words: BS4 Enzyme, Palm Kernel Cake, Rice Bran, Egg Production, Egg Quality

INTRODUCTION

Exogenous enzymes are widely used as feed additives in poultry diet nowadays. Although some enzymes such as amylase, are produced naturally for digestion of feed in the gastro-intestinal tract of poultry, supplementation of enzymes are meant to complement the endogenous enzymes to maximize nutrients utilization from feed, hence improve the feed efficiency.

Some feed ingredients were known to contain anti-nutritional factors which caused poor digestibility for monogastric animal. Two feed ingredients which are produced abundantly in Indonesia and commonly included in poultry diet, i.e., rice bran and palm kernel cake (PKC) also contain some anti-nutritional factors. Rice bran contains high crude fibre (13.0%) which

consists of cellulose, hemicellulose and lignin that are poorly digested by poutry (Gallinger et al. 2004; Batal & Dale 2012). According to Sundu et al. (2006) PKC contains 21-23% crude fibre. In more detail, Knudsen (1997) showed that PKC contains high (42%) non-starch polysaccharides (NSPs) in which, 33.6% was insoluble NSPs. The high NSPs in the diet is considered as antinutritional factors since it can have negative effects on the digestibility and rate of absorption of carbohydrates, fat and protein or amino acids in poultry (Choct et al. (2010).

Inclusion of more than 10% rice bran in broiler diets (Gallinger et al. 2004) or laying hens diet (Samli et al. 2006) or more than 20% in laying quail diet (Abeyrathna et al. 2014) have shown significant reduction on their performances. Chong et al. (2008) reported that inclusion of 12.5 % PKC in the diet significantly impaired the feed convertion ratio of laying hens and inclusion of 25% PKC impaired the FCR and eggs size, although the level of egg production was not affected. Sinurat et al. (2011) also showed that inclusion of 20% PKC in the diet, slightly reduced the egg production of laying hens.

Supplementation of exogenous enzyme, especially carbohydrases into poultry diet have been reported successfully to degrade NSPs in poultry diet and the effect of enzyme supplementation is more obvious when cereal with poor digestibility is present in the diet (Bedford 2000). Altough supplementation of enzymes also capable of improving the performance and intestinal physiological parameters of laying hens fed with high digestible feedstuffs such as corn-soy (Lee et al. 2014).

An enzyme complex, produced by *Eupenicilium javanicum* BS4 has been developed in our laboratory. The enzyme was aimed to improve nutrients digestibility of local feedstuffs with low quality such as palm oil by products (palm oil sludge and palm kernel cake). The enzyme was produced by cultivating *Eupenicillium javanicum* on coconut meal and consist of β-mannanase, CMCase (cellulase), β-mannosidase, β-glucosidase and α-galactosidase (Haryati et al. 1997) which effectively digest cellulose and hemicellulose in palm kernel cake and palm sludge (Purwadaria et al. 2004). The effectivity of the enzyme to improve, metabolisable energy, dry matter and protein digestibilities of palm oil sludge (Sinurat et al. 2007; Sinurat et al. 2009; Pasaribu et al. 2009) and palm kernel cake (Sinurat et al. 2013; Sinurat et al. 2014; Sinurat et al. 2015) have been reported. The present study was designed to test the effectivity of the enzyme (BS4 enzyme) in improving the performance of laying hens when the feed was formulated to consist different feedstuffs, generally known to have low (maize) or high (PKC and rice bran) anti-nutritional levels.

MATERIALS AND METHODS

Three (3) diets consist of feedstuffs which are known to have different ANFs level, i.e., maize (low ANFs level), 30% rice bran (high ANFs level) and 20% PKC (high ANFs level) were formulated with similar nutrient values (iso- ME, Iso- protein and digestible amino acids) to meet the nutrient requirement of laying hens. The nutrient contents of the diet were: 17% crude protein, 2750 kcal/kg ME, 3.9% calsium (Ca), 0.38% available phosphorous (Av. P), 0.740% digestible lysine, 0.440% digestible methionine, 0.635% digestible metionine + cystine, 0.160% digestible tryptophan and 0.523% digestible threonine. The formula and the composition of the diets are presented in Table 1. Each diet was either supplemented or not with BS4 enzyme. The enzyme was produced by *Eupenicillium javanicum* using coconut cake as substrate (Rakhmani et al. 2015). The level of enzyme supplemented was based on the results of previous experiment, i.e., 150 Unit/kg substrate (Sinurat et al. 2014; Sinurat et al. 2015).

One hundred and fourty four (144) Isa brown pullets, aged 16 weeks were allocated randomly, reared in individual wire cages and fed similar diet (pre-lay diet) for three (3) weeks. Each four (4) birds were provided with one feeder and therefore considered as one replicate. The birds were then fed with experimental diets from age 19 weeks to the end of experiment (37 weeks). Each treatment was fed to 24 hens (6 replicates with 4 birds/replicate). The production parameters were start measured a week later (age 20 weeks) for the next 18 weeks. Variables measured were body weight changes, feed intake, hen-day egg production, feed convertion ratio (calculated by dividing g feed intake by g egg produced) and egg quality (egg shell thickness and yolk color score). Body weight changes were measured by weighing the birds before and after the trial. Feed intakes were measured weekly. Eggs produced were collected and weighed daily. After 10 weeks treatment (birds aged 29 weeks), the egg quality were measured. All data were subject to analyses of variance in a 2 X 3 factorial design, i.e., 2 levels of enzyme supplementation (with or without enzyme supplementation) X 3 feedstuffs inclusion in the diet (maize, PKC and rice bran). The comparisons between treatment means were calculated by Duncan's multiple range test when the ANOVA was significant or $P<0.05$ (Steel & Torrie 1997).

Table 1. Composition of experimental diets with different kind of feedstuffs inclusion

Feed ingredients	Source of ANFs		
	Maize	Palm kernel cake	Rice bran
Rice bran	0	0	**30**
Palm kernel cake	0	**20**	0
Maize	62.58	45.32	36.40
Soybean meal	22.91	9.23	12.15
Meat and bone meal 50	0.78	5.7	4.87
Corn gluten meal 60	2.5	6.5	5
Limestone	9.39	8.55	8.78
Cooking oil	0	3.75	1.89
DL-methionine	0.17	0.16	0.19
L-lysine	0	0.23	0.17
L-threonine	0	0.01	0
Sodium bicarbonate	0.1	0.1	0.1
Salt	0.25	0.25	0.25
Vitamin premix	0.03	0.025	0.025
Mineral premix	0.05	0.05	0.05
Mono calcium phosphate (MCP)	1.15	0	0
Choline chloride	0.09	0.09	0.09
Tryptophan	0	0.04	0.04
Total	100	100	100
Nutrient contents			
Dry matter (%)	89.8	90.1	89.7
Crude fibre (%)	2.48	4.64	5.46
Metabolisable Energy (ME), kcal/kg	2750	2744	2750
Crude Protein (%)	17.70	17.70	17.70
Calcium (Ca) (%)	3.90	3.90	3.90
Available phosphorous (Av. P) (%)	0.38	0.38	0.38
Digestible lysine (%)	0.740	0.740	0.740
Digestible methionine (%)	0.440	0.442	0.438
Digestible methionine + Cystine (%)	0.635	0.630	0.630
Digestible tryptophan (%)	0.160	0.160	0.160
Digestible threonine (%)	0.523	0.523	0.523

RESULT AND DISCUSSION

The effect of feeding different feedstuffs and enzyme supplementation on the performance of the laying hens are presented in Table 2. Results of analysis of variance did not show any significant (P>0.05) effect of interaction between feedstuffs (F) and enzyme supplementation (E) on the performance parameters (body weight changes, feed intake, HD egg production, egg weight and feed convertion ratio or FCR) during

the 18 weeks of trial. Therefore the performance data are presented only to show the main effects of the treatment. Lack of significant interactions effect between E x F means that the effect of enzyme supplementation is applicable to all feedstuffs (maize, PKC and rice bran) used in this experiment. The mortality of the birds during the 18 weeks trial was very few (only 2 birds). The mortality was not considered as the effect of the treatments and therefore was not analysed statistically.

Supplementation of BS4 enzyme complex did not significantly ($P>0.05$) affect body weight changes, feed intake and egg weight, but significantly ($P<0.05$) influenced the HD egg production and feed convertion ratio (FCR) of laying hens (Table 2). Although the body weight gain is not the main output of laying hens, the measurement of body weigh changes is important as an indication of nutrients adequacy for the hens. The result showed that the BS4 enzyme supplementation did not cause nutrients imbalance in the laying hens diet. This is also supported by the feed intake data which showed no effect due to enzyme supplementation. The BS4 enzyme has been shown to increase the metabolisable enery (ME) but also the protein digestibility of PKC (Sinurat et al. 2011; Sinurat et al. 2013) and the amino acids digestibility of fermented-PKC (Sinurat et al. 2014).

Supplementation of the BS4 enzyme increased hen day egg productions significantly ($P<0.05$). Since the enzyme supplementation did not affect the feed intake, the increase in HD egg production (from 87.26% to 90.03%) could be the effect of improvement in overall nutrients (energy, protein and amino acids) utilization by the hens as reported previously (Sinurat et al. 2011; Sinurat et al. 2013; Sinurat et al. 2014). As a result, the BS4 enzyme showed a significant ($P<0.01$) improvement in the feed efficiency or FCR. Chong et al. (2008) reported that commercial enzyme supplementation did not show improvement in egg production of laying hens fed PKC diet, but improve the FCR. Different kind and activities of enzyme used in both studies explained this discrepancy.

Although the mortalities of hens fed diet without enzyme was higher (2.7%) than those fed enzyme diet (0%). It is not considered as the effect of enzyme supplementation. Mortalities about 2% during the period of the experiment (age 19 to 37 weeks old) are common for Isa brown laying hens (Hendrix-Genetic 2007).

Supplementing exogenous enzymes into poultry diet have been reported by many authors in order to minimize the ANFs in poultry diet (Ravindran 2013). Based on some researches finding, Ravindran (2013) suggested that multiple enzymes may give a better results in improving nutrients utilization in poultry diet as compared to single enzyme. The presence of multi enzyme (more than one enzyme) may give additive or synergistic effect on nutrient utilization and animal performances. Saenphoom et al. (2013) reported that enzyme treatment effectively hydlrolyzed the fibre components of PKC and increase the TME of PKC in broilers.

As shown in Table 2, the body weight changes of laying hens were all positive or all birds gained some weight. The body weight changes were neither affected ($P>0.05$) by inclusion of different feedstuffs nor by enzyme supplementation. Body weight gain is not the main output of laying hens. However, body weigh changes can be a reflection of nutrients imbalance in the diet. Imbalances of dietary amino acids (methionine and cystine) have been reported to have a quadratic effect on body weight gain of laying hens (Narvaez-Solarte et al. 2005). Similar body weight changes in this experiment showed that inclusion of different feedstuffs and supplementation of BS4 enzyme did not affect the amino acids balance in the diet. This might be due to diet formulation applied in this experiment, i.e., based on digestible amino acids rather than on total amino acids.

The weekly egg production of laying hens for each treatment and comparison with the standard is provided in Figure 1. The rate of egg production was normal, when compared to Isa brown standard performance. In general, the egg production pattern and the average HD egg production of laying hens in this experiment was similar to the standard of Isa brown hens. The average HD egg production during the same period (age 20 to 37 weeks) according to Hendrix-Genetic (2007) was 88.6%.

Inclusion of 20% PKC in laying hens diet showed significantly ($P<0.05$) higher hen-day egg production as compared to inclusion of maize or rice bran, although all diets were formulated to have similar nutrient values. Similar finding was also reported by Sinurat et al. (2011) when 5% PKC but not 20% PKC included in the diet. Zanu et al. (2012) also reported an improvement in HD egg production when 5% or 10% PKC was included in laying hens diet. It is possible that inclusion of PKC in the diet has contributed some prebiotic that improve the health of gastro intestinal tract of the hens, improve nutrients absorbtion and and subsequently increase the egg production. PKC is known as a good source of β-mannan or its hydrolysed product oligomannan which can be functioned as prebiotics (Utami et al. 2013; Jahromi et al. 2016) which may improve the immune system of the birds. Inclusion of 20% PKC in broiler's diet has been reported to be as effective as commercial prebiotic in reducing intestinal bacteria (*Escherichia coli*) populations (Navidshad et al. 2015). Jahromi et al. (2016) also reported that supplementation of oligosaccharide (1 g/kg diet) extracted from PKC increased the populations of beneficial bacteria

Table 2. Production performance of laying hens as affected by feeding different feedstuffs and enzyme supplementation

Feedstuff	Body weight change, (g/bird)	Egg production % HD	Egg weight (g)	Feed intake (g/d)	FCR	Mortality (%)
Effect of different feedstuffs						
Maize	285.3	86.77[a]	56.50	114.2[a]	2.424	0
Palm kernel cake	264.3	91.43[b]	55.99	117.4[b]	2.326	2.1
Rice bran	294.1	87.73[a]	55.89	113.1[a]	2.408	2.1
Effect of enzyme supplementation						
- Enzyme*	290.6	87.26[a]	56.13	114.7	2.453[b]	2.7
+ Enzyme*	271.8	90.03[b]	56.12	115.1	2.319[a]	0
Level of significance (P)						
Feedstuff (F)	0.647	0.009	0.601	0.001	0.147	n.a**
Enzyme (E)	0.485	0.028	0.078	0.672	0.004	n.a
E x F	0.940	0.515	0.667	0.661	0.221	n.a

* + means enzyme supplemented; - means no enzyme supplemented
** n.a = not analysed statistically
Different superscript in the same column and treatment means significantly different (P<0.05)

(*Lactobacillus, Bifidobacterium* and *Enterococcus*) and suppressed the populations of pathogenic (*E. coli* and *Enterobacter*) bacteria in the cecum of broiler chickens.

Feed intake of laying hens was significantly (P<0.01) affected by inclusion of different feedingstuff in the diet. The feed contained PKC were consumed more than those contained maize and rice bran. Chong et al. (2008) also reported an increase in feed intake when 12.5% or 25% PKC were included in the diet of of laying hens. However, this was not found by Sinurat et al. (2011) and Zanu et al. (2012). The incresase in the egg production as described earlier could be speculated as the effect of the increase in feed intake, since the feed convertion ratio (FCR) was not significantly (P>0.05) affected by inclusion of different feedstuff in the diet. It is commonly known that feed intake in poultry is dominantly controlled by energy level in the diet. Since all diet in this experiment was formulated iso- energy, the mechanism of feed intake increases in this case is not clearly understood. Whether the presence of prebiotic or oligosaccharide contributed by PKC in the diet stimulate the feed intake or not, needs to be investigated further. Navidshad et al. (2015) showed a slightly (2%) increase in feed intake of broilers when 20% of PKC was included in the diet.

The inclusion of different feedstuff in the diet did not significantly (P>0.05) affect the egg weight, FCR and mortalities. Feedstuffs contain high ANFs (crude fibre or NSPs) are included in the poultry diet in limited amount. In general, feed mills in Indonesia only included maximum of 20% rice bran and 5% PKC in laying hens diet. The present study showed that inclusion of 30% rice bran or 20% PKC did not impair the productivity of laying hens. Other reports showed that inclusion of 10% (Sinurat et al. 2011; Zanu et al. 2012), 20% (Perez et al. 2000), or 25% PKC (Chong et al. 2008) in the diet can support a good productivity performance of laying hens. Therefore, in countries with high production of rice bran and or PKC such as Indonesia, it is encouraged to use these feedstuffs as it may reduce the cost of production.

The effect of inclusion of different feedstuffs and enzyme supplementation on the egg quality is presented in Tabel 3. The egg shell thickness was significantly (P<0.01) affected by inclusion of different feedstuffs but not by enzyme supplementation in the diet nor by the interaction between the two factors. Inclusion of rice bran significantly produced thicker egg shell than others. According to Zita et al. (2009) egg shell thickness of Isa brown was 37-38 μm. Nutritionally, egg shell thickness were mainly influenced by calcium (Ca), available phosphorus (Av. P) and D vitamin. All diets have been formulated to to contain similar levels of those nutrients (Table 1). Rice bran is known to contain high P levels but very low Av. P. Improper balance between Ca and Av. P may affect the egg shell thickness. Although all diets were formulated to contain similar available P (0.38%), it might be that the calculated value of the Av. P of rice bran was over estimated and the diet with rice bran was lower than expected. Abubakar et al. (2007) also reported an increase in egg shell thickness of laying hens when 30% rice bran was included in the diet as compared to the control (without rice bran).

As shown in Table 3, the egg yolk color score was significantly (P<0.01) affected by the interaction

between feeding different feedstuffs (F) and enzyme supplementation (E). Statistical analyses revealed that supplementation of BS4 enzyme in maize- feed or rice bran did not affect the egg yolk color significantly (P>0.05). However, enzyme supplementation in PKC-feed increased the egg yolk color score significantly (P<0.05). Inclusion of PKC in the diet without enzyme supplementation, significantly (P<0.05) produced higher egg yolk color score as compared to others. Previous study also reported an increase in egg yolk color score when PKC (Zanu et al. 2011), or enzyme supplemented fermented-PKC (Sinurat et al. 2014) was included in laying hens diet.

CONCLUSION

The results of this study conclude that BS4 enzyme is effective in improving egg production and feed efficiency (FCR) when supplemented into feed of laying hens contains either maize, 30% rice bran and 20% palm kernel cake (PKC). Supplementation of BS4 enzyme into laying hens diet did not influence the body weight change, feed intake, mortalities, egg weight and egg shell thickness. Laying hens fed diet with 20% PKC produced more eggs than those fed diet with maize or rice bran and supplementation of BS4 enzyme into the diet improved the egg yolk color score fed the PKC diet but no effect on maize or rice bran diet.

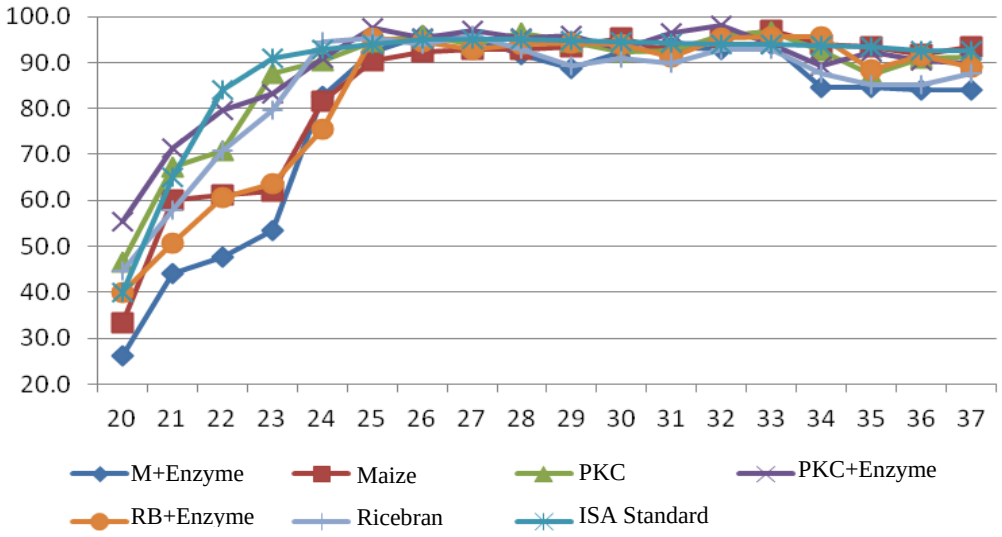

Figure 1. Weekly egg production of laying hens fed with different feedstuffs and enzyme supplementation.

Table 3. Egg quality of laying hens as affected by feeding different feedstuffs and enzyme supplementation

Feedstuff	Enzyme**	Egg shell thickness*, µm	Yolk color score*
Maize	-	45.3[a]	4.6[d]
Maize	+	45.4[a]	4.8[cd]
Palm kernel cake	-	47.2[a]	5.4[b]
Palm kernel cake	+	46.4[a]	6.2[a]
Rice bran	-	48.7[b]	5.0[c]
Rice bran	+	47.9[b]	4.8[cd]
Level of significance (P)			
Feedstuff (F)		0.008	0.001
Enzyme (E)		0.405	0.013
F x E		0.907	0.001

* Values with different letters within the same column different significantly (P<0.05)
** + means enzyme supplemented; - means no enzyme supplemented

ACKNOWLEDGEMENT

The authors appreciate all the technicians who helped to produce the enzyme and looking after the animal. Those were Mr Helmi Hamid, Mrs Emi Sujatmika, Mr Kadiran and Mr Agus.

REFERENCES

Abeyrathna HMWN, Atapattu NSBM, Gunawardane WWDA. 2014. Effects of the level of dietary rice bran with or without phytase on performance and egg parameters of laying Japanese quail. Trop Agric Res. 26:39-47.

Abubakar A, Tukur HM, Sekoni AA, Hassan WA. 2007. Performance and egg quality characteristics of laying birds fed diets containing rice bran with and without yeast supplementation. Asian J Anim Sci. 1:1-9.

Batal A, Dale N. 2012. Ingredient analysis table: 2011 Edition. Feedstuffs, 15 September 2010. [accessed August 9th 2014]. Available from: http://fdsmagissues. feedstuffs.com.

Bedford MR. 2000. Mechanism of action and potential environmental benefits from the use of feed enzymes. Anim Feed Sci Tech. 53:145-155.

Choct M, Derrsjant-Li Y, McLeish J, Peisker M. 2010. Soy oligosaccharides and soluble Non-starch polysaccharides: A Review of digestion, nutritive and anti-nutritive effects in pigs and poultry. Asian-Aust J Anim Sci. 23:1386-1398.

Chong CH, Zulkifli I, Blair R. 2008. Effects of dietary inclusion of palm kernel cake and palm oil and enzyme supplementation on performance of laying hens. Asian-Aust J Anim Sci. 21:1053-1058.

Gallinger CI, Suarez DM, Irazusta A. 2004. Effects of rice bran inclusion on performance and bone mineralization in broiler chicks. J Appl Poult Res. 13:183-190.

Haryati T, Purwadaria T, Darma J, Tangendjaja B. 1997. Production of extracellular glycosidase by *Eupenicillium javanicum* and *Aspergillus niger* NRRL 337 on the coconut meal substrate. Proceeding Second Conference on Agricultural Biotechnology. Jakarta (Indones): IAARD. p. 517-522.

Hendrix-Genetics. 2007. Performance of Isa Brown final product. [accessed March 22th 2012]. http://www. hendrix-genetics.com.

Jahromi MF, Liang JB, Abdullah N, Goh YM, Ebrahimi R, Shokryazdan P. 2016. Extraction and characterization of oligosaccharides from palm kernel cake as prebiotic. Bio Resources. 11:674-695.

Knudsen KEB. 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim Feed Sci Tech. 67:319-338.

Narvaez-Solarte W, Rostagno HS, Soares PR, Silva MA, Velasquez LFU. 2005. Nutritional requirements in methionine + Cystine for White- egg laying hens during the first cycle of production. Int J Poult Sci. 4:965-968.

Navidshad B, Liang JB, Jahromi MF, Akhlaghi A, Abdullah N. 2015. A comparison between a yeast cell wall extract (Bio-Mos®) and palm kernel expeller as mannan-oligosac-charides sources on the performance and ileal microbial population of broiler chickens. Ital J Anim Sci. 14:3452.

Pasaribu P, Sinurat AP, Purwadaria T, Ketaren P. 2009. Peningkatan nilai gizi solid heavy phase sebagai pengganti jagung dalam pakan unggas. JITV. 14:167-176.

Perez JF, Gernat AG, Murillo JG. 2000. The effect of different levels of palm kernel meal in layer diets. Poult Sci. 79:77-79.

Purwadaria T, Nirwana N, Ketaren PP, Pradono DI, Widyastuti Y. 2003. Synergistic activity of enzymes produced by *Eupenicillium javanicum* and *Aspergillus niger* NRRL 337 on palm oil factory wastes. Biotropia. 20:1-10.

Rakhmani SIW, Pangestu Y, Sinurat AP, Purwadaria T. 2015. Carbohydrate and protein digestion on palm kernal cake by Mannanase BS4 and papain cocktail enzymes. Indones J Anim Vet Sci. 20:268-274.

Ravindran V. 2013. Feed enzymes: The science, practice, and metabolic realities. J Appl Poult Res. 22:628-636.

Saenphoom P, Liang JB, Ho YW, Loh TC, Rosfarizan M. 2013. Effects of enzyme treated palm kernel expeller on metabolizable energy, growth performance, villus height and digesta viscosity in broiler chickens. Asian-Aust J Anim Sci. 26:537-544.

Samli HE, Senkoylu N, Akyurek H, Agma A. 2006. Using rice bran in laying hen diets. Cent Eur Agric. 7:135-140.

Sinurat AP**,** Purwadaria T, Bintang IAK, Pasaribu T, Manurung BP, Manurung N. 2008. Substitution of corn with enzymes treated palm oil sludge in laying hens diet. Proceedings XXIII World's Poultry Science Congress. Brisbane (Australia): World's Poultry Science Association.

Sinurat AP, Purwadaria T, Pasaribu T, Ketaren P. 2011. Performances of laying hens fed with enzyme supplemented palm kernel cake diets. Proceedings 9th Asia Pacific Poultry Conference. Taipeh (Taiwan): World's Poultry Science Association.

Sinurat AP, Purwadaria T, Pasaribu T. 2013. Peningkatan nilai gizi bungkil inti sawit dengan pengurangan cangkang dan penambahan enzim. Indones J Anim Vet Sci. 18:34-41.

Sundu B, Kumar A, Dingle J. 2006. Palm kernel meal in broiler diets: Effect on chicken performance and health. World Poult Sci J. 62:316-325.

Utami W, Meryandini A, Wiryawan KG. 2013. Characterization of bacterial mannanase for hydrolyzing palm kernel cake to produce manno-oligosaccharides prebiotics. Media Peternakan. 36:192-196.

Zanu HK, Abangiba J, Arthur-Badoo W, Akparibo AD, Sam R. 2012. Laying chickens' response to various levels of palm kernel cake in diets. Int J Livest Prod. 3:12-16.

Zita L, Tumova E, Stolc L. 2009. Effects of genotype, age and their interaction on egg quality in brown-egg laying hens. Acta Vet BRNO. 78:85-91.

In Vitro Protein Digestibility and Fermentability of Mulberry (*Morus alba*)-Leucaena Foliage Mixed Feed

Yulistiani D[1], Jelan ZA[2], Liang JB[3]

[1]*Indonesian Research Institute for Animal Production, PO Box 221 Bogor 16002, Indonesia*
[2]*Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor 43400, Malaysia*
[3]*Institute of Tropical Agriculture, Universiti Putra Malaysia, Serdang, Selangor 43400, Malaysia*
E-mail: dwiyulistiani@yahoo.com

(received 22-12-2015; revised 27-01-2016; accepted 16-02-2015)

ABSTRAK

Yulistiani D, Jelan ZA, Liang JB. 2016. Kecernaan protein *in vitro* dan fermentabilitas pakan campuran hijauan murbei dan leucaena. JITV 21(1): 9-18. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1352

Penelitian dilakukan untuk mengevaluasi pengaruh campuran hijauan murbei dengan leucaena terhadap kecernaan protein dan produksi VFA (asam lemak volatile) yang diukur secara *in vitro* produksi gas. Hijauan murbei dicampur dengan dua varietas leucaena (*Leucaena leucocephala* hibrida dan *Leucaena leucocephala* lokal) pada tiga aras (0, 25 dan 50%). Penelitian dilakukan menggunakan rancangan acak lengkap. Tepung hijauan murbei, leucaena dan campuran hijauan murbei-leucaena diinkubasi dalam gelas syringe selama 24 jam. Parameter yang diamati adalah produksi gas, kecernaan sejati *in vitro* bahan kering (IVTDMD), kecernaan N *in vitro* (IVND), dan produksi VFA. Hasil penelitian menunjukan bahwa suplementasi hijauan leucaena pada hijauan murbei menurunkan produksi gas, kecernaan bahan organik, dan kecernaan protein dalam rumen buffer. Campuran hijauan murbei dengan leucaena hibrida pada rasio 50% menghasilkan kecernaan protein terendah dalam rumen buffer dibandingkan dengan perlakuan yang lain. Namun demikian terjadi peningkatan kecernaan protein dalam pepsin HCl yang merupakan indikator prakiraan kecernaan protein dalam usus. Total produksi gas terjadi penurunan pada campuran hijauan murbei leucana yang diikuti dengan penurunan produksi total VFA. Suplementasi leucaena hibrida pada murbei dengan rasio 1 : 1 paling efektif untuk menurunkan kecernaan protein ruminal dan meningkatkan kecernaan protein yang diinkubasi pada pepsin HCl. Dapat disimpulkan bahwa pencampuran leucaena hybrid mampu memproteksi degradasi protein murbei di dalam rumen.

Kata Kunci: Murbei, Leucaena, Kecernaan Protein, *In Vitro*

ABSTRACT

Yulistiani D, Jelan ZA, Liang JB. 2016. *In Vitro* protein digestibility and fermentability of mulberry (*Morus alba*)-Leucaena foliage mixed feed. JITV 21(1): 9-18. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1352

This experiment was carried out to determine the effect of mulberry-leucaena foliage mixed feed on protein digestibility and VFA production using an *in vitro* gas production study. Mulberry was mixed with one of 2 leucaena varieties (*Leucaena leucocephala* hybrid and *Leucaena leucocephala* local) at 3 levels (0, 25 and 50%). Study was conducted in completely randomized design. Mulberry foliage, leucaena and mixtures of mulberry-leucaena were incubated for 24 hours in glass syringes. Parameter recorded were gas production, *in vitro* true dry matter digestibility (IVTDMD), *in vitro* N digestibility (IVND) and VFA production. Results of the study showed that supplementation of leucaena to mulberry decreased IVOMD, gas production and protein digestibility in the rumen buffered medium. The mixture of mulberry and leucaena hybrid at ratio 50% resulted in the lowest IVND than other treatment. However it increased protein digestibility in acid pepsin incubation as an estimate of protein availability in intestine. Gas production decreased in mulberry leucaena mixtures was followed by decreased total VFA production. Tannin derived from leucaena hybrid supplementation to mulberry at ratio 1 : 1 was most effective to decrease protein digestion in the rumen and to increase protein digestibility in acid pepsin incubation. In conclusion mixing of hybrid leucaena hybrid with mulberry foliage was able to protect protein degradation from mulberry in the rumen.

Key Words: Mulberry, Leucaena, Protein Digestibility, *In Vitro*

INTRODUCTION

The key of successful and sustainable ruminant farming in tropical conditions is dependent on the attempt to formulate reliable cheap feeding strategy based on resource availability. This is due to the scarcity and fluctuation in quantity and quality of year round supply of conventional feeds. The situation is exacerbated by the increase human population and decrease land availability for forage crop production that cause ruminants feeding depends on crop residue and agricultural by products with "low nutritional quality". One of the methods to maximize the utilization of fibrous agricultural residues is by supplementing essential nutrients in the basal feed to correct the nutrients imbalances. Several

supplementation strategies have been developed using commercial concentrate, this supplementation could improve crop residues digestibility, intake and animal performance. However, although the above method is effective, the using of concentrate by small farmers is limited by cost particularly when it is imported. The high cost of imported concentrate led to seeking for alternative locally available forages for ruminants feed. This effort has practical implication because it is easy to adopt by small scale farmers. Tree foliages, shrubs and agro-industrial by-products are important in animal production in the tropics because they do not compete with human food and can provide significant protein supplements (Makkar 2003). Recently, there is increasing interest on the use Mulberry as ruminants feed due to its biomass production potency, palatability and nutritive value. Supplementation of pelleted mulberry leaves was able to improve rumen fermentation and nutrient digestibility of cattle fed on rice straw basal diet (Huyen et al. 2012), recently Yulistiani et al. (2015) also reported that in urea treated rice straw basal diet, mulberry supplementation can replace urea and rice bran as energy and protein sources. However, protein degradability of mulberry was very high. After 24 hours incubation, the protein of mulberry was degraded more than 80% in the rumen regardless of dietary treatments of the sheep (Yulistiani et al. 2008). Gemeda & Hassen (2015) also reported that, *in vitro* incubation of mulberry (*Morus alba*) produced highest NH_3N among the tropical browse plants from South Africa, indicating that protein in mulberry was highly degradable in the rumen resulting in the loss of valuable essential amino acid sources for host animals (Bach et al. 2005)

There are many methods to reduce protein feed degradability in the rumen Saddul et al. (2004) reported that heating of mulberry foliage could reduce its protein degradability. Compared to other treatments, Protein protection using tannin is better and give positive response, Getachew et al. (2008) reported addition of quebraco tannin or tannin acid ranges from 5-15% reduced *in vitro* rumen degradability of protein in alfalfa forage.

Leucaena, one of leguminous trees can be used as tannin source to protect protein degradability of soy bean meal (Cortes et al. 2009). Hybrid *Leucaena* developed in Malaysia has been reported to have high content of condensed tannin (13%) (Khamseekhiew 2006) and moderate amount (2.0-4.5% DM) of condensed tannin has a beneficial effect on protein metabolism in ruminants and give better nutritional value of tree fodder species (Comacho et al. 2010). Condensed tannin decreased rumen degradation of dietary protein and increased absorption of amino acids in the small intestine (Barry & McNabb 1999). The mixture of *mulberry*, *Leucaena* and *Tectona grandis* leaves could result in increase rumen escape protein (Anbarasu et al. 2004), which can be utilized by the host animals. The objectives of this study were to evaluate the digestibility and protein degradability in mixture of *Leucaena* and mulberry by *in vitro* gas production technique and to determine the best ratio of *Leucaena* to mulberry in reducing protein degradation in the rumen.

MATERIALS AND METHODS

Feed

Mulberry foliage

Mulberry grown at the experimental plot of the Department of Animal Science, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, was harvested after about 5-7 weeks re-growth. Foliage was air-dried under shed for 3 days, chopped then ground using hammer mill and passed through a 1mm sieve.

Leucaena foliage

The foliage was harvested at 8 weeks of re-growth (from previous cutting), oven-dried at 45°C for 2 days, chopped, ground using hammer mill and passed through a 1mm sieve. *Leucaena leucocephala* local variety and *Leucaena leucocephala* hybrid variety contained condensed tannin (CT) 9.6 and 13.1% respectively (Khamseekhiew 2006), while mulberry foliage contained CT 0.16% (Saddul 2005).

Preparation of diets

Mulberry foliage was mixed with each of two varieties of *Leucaena* (hybrid or local) at 2 levels: 25% and 50% on DM basis as shown in Table 1 and the nutrient composisition of the diets presented in Table 2.

In vitro OM digestibility

A 24 hours gas production test was carried out to determine truly degradable fermented substrates (*in vitro* true dry matter degradability/IVTDMD). In this incubation, 500 mg sample was incubated in 40 ml of medium. The medium was prepared according to Makkar et al. (1997). Each diets treatment was incubated in 4 syringes as replication. Gas volume was recorded at 2, 4, 6, 8, 10 and 24 h incubation. After terminating the incubation, five ml of supernatant from each syringes was taken for volatile fatty acid (VFA) analysis, prior the residue in the syringe was transferred into a 600 ml spoutless beaker. The syringe was washed with a total of 70 ml of NDS solution. The procedure of

Table 1. List of experimental diets

Type of diet	Ratio (%)			Calculated CT content of mixed feed (%)
	Mulberry	*Leucaena* hybrid	*Leucaena* local	
Mulberry (M)	100	0	0	0.16
Leucaena leucocephala hybrid (LH)	0	100	0	9.60
Leucaena leucocephala local (LL)	0	0	100	13.10
M/LH (1 : 1)	50	50	0	6.45
M/LL (1 : 1)	50	0	50	4.81
M/LH (3 : 1)	75	25	0	3.13
M/LL (3 : 1)	75	0	25	2.46

LL: *Leucaena leucocephala* local variety; LH: *Leucaena leucocephala* hybrid variety; M: Mulberry

Table 2. Nutrient composisition of experimental feeds

Feed ingredients	Chemical composition (% DM)				
	DM	OM	CP	NDF	ADF
Mulberry	92.2	91.7	18.9	41.5	25.4
Leucaena leucocephala hybrid (LH)	93.6	95.7	21.5	45.1	28.3
Leucaena leucocephala local (LL)	93.0	95.1	21.3	44.5	29.0
M/LH (1 : 1)	92.3	93.7	20.2	43.3	26.8
M/LL (1 : 1)	92.6	93.4	20.1	43.0	27.2
M/LH (3 : 1)	93.1	74.1	25.4	38.2	19.0
M/LL (3 : 1)	92.4	92.5	19.5	42.2	26.3

M/LH (1 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 50 : 50%
M/LH (3 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 75 : 25%
M/LL (1 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 50 : 50%
M/LL (3 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 75 : 25%

van Soest (1991) was then applied by refluxing the incubation residue for 1.0 h and filtering the undigested matter on pre-tared filter crucibles. True digested of dry matter was calculated as the weight of substrate incubated minus the weight of the residue after NDS treatment. Rumen fluid was obtained from rumen-cannulated cattle maintained on roughage concentrate diet at the ratio of 60 : 40 DM.

Determination of *in vitro* N digestibility

Determination of *in vitro* N digestibility (IVND) was conducted on the samples using a modified Tilley & Terry (1963) two-stage digestion method (Palmer & Jones 2000). 0.5 g DM of each sample was weighed into calibrated glass syringes, followed by addition of 40 ml of rumen Fluid: buffer (1 : 4). There were 2 sets of 4 replicates of each treatment together with four blanks in separate tubes that received no sample were incubated. The syringes were incubated in water bath at 39°C for 24 h. Then the content of the syringes was centrifuged for 20 min at 2500 g. Supernatant of 3 ml was taken for VFA analysis. The remaining supernatant was decanted and 40 ml of distilled water was added. The suspension was thoroughly mixed using a vortex mixer and then centrifuged again for 20 min, the supernatant again discarded, and the process repeated 3 times. Each tube from one set of 4 replicates then added with 40 ml of acid pepsin (2 g of 1 : 10,000 pepsin in 1 l of 0.1 M HCl), thoroughly mixed then incubated at 39°C for 24 h. After incubation, the mixture was centrifuged for 20 min, the supernatant discarded, and the residue dried at 65°C for 48 h prior to weighing. Another one set of 4 replicates after being centrifuged was oven dried at 65°C for 48 h prior to weighing. The calculation of IVND in the rumen medium (X).

$$X = \frac{A - (B-D)}{A} \times 100$$

whereas calculation of total IVND (Y) was:

$$Y = \frac{A - (C-D)}{A} \times 100$$

A = N in sample before incubated
B = N in residue after samples incubated in the rumen buffered medium
C = N residue after samples incubated in acid pepsin solution
D = N residue of the blank after incubation

$$P = \frac{R}{A} \times 100$$

where:

P = The IVND in the acid pepsin
R = total IVND (X) - IVND in the rumen (Y)

The predicted N digestibility was also calculated from the incubation of individual feeds which then extrapolated, this calculation was done to see the synergistic effect of mulberry and leucaena mixture.

Chemical analyses

The feed samples were analyzed for DM, OM and CP according to the procedures of AOAC (2000). ADF and NDF were determined using the method of Van Soest et al. (1991).

The volatile fatty acid (VFA) and molar proportions of acetic, propionic and butyric acids of supernatant from incubated samples were determined by gas chromatography (Model G1540N, Agilent Technologies, USA) fitted with a flame ionization detector (FID) and a packed column 5% Thermon-3000, Shincarbon A 60/80. Nitrogen was used as the carrier gas at 40 ml/min and the oven temperature was maintained at 220°C. Injection and FID temperature were fixed at 260°C.

Statistical analysis

The experiment was carried out in a completely randomized design and data were analysed using Anova of SAS package version 9.1 (2002). Means were compared by Duncan's multiple range test.

RESULT AND DISCUSSION

Result

The effect of *Leucaena* mixed with mulberry on gas production, IVDMD and amount of substrate fermented is shown in Table 3. Gas production and true organic matter digestibility were significantly ($P<0.05$) lower in mulberry mixed with *Leucaena* than mulberry alone. However, the the amount drry matter being fermented was not significantly decreased when mulberry was mixed with hybrid *Leucaena* at 50% level. Hybrid *Leucaena* had significantly ($P<0.05$) higher gas production, digestibility and amount of substrate fermented than the local variety of *Leucaena*.

Table 4 shows the effect of mulberry mixed with *Leucaena* on protein digestibility in rumen buffer media, acid pepsin solution and total protein digestibility. Protein degradation in the buffered rumen media was significantly decreased in mixture of mulberry and *Leucaena* compared to mulberry alone. The decrease of protein degradation was highest in mulberry mixed with 50% hybrid *Leucaena* M/LH (1 : 1). The decrease of protein degradation in rumen buffered media caused the increased protein degradation in the acid pepsin incubation. The M/LH (1 : 1) mixture had the highest protein digestion in acid pepsin. However, other mixtures, their protein digestion was significantly lower M/LH (3 : 1) and M/LL (3 : 1) or similar M/LL (1 : 1) to *Leucaena*. Total protein digestibility was significantly ($P<0.05$) decreased in mulberry mixed *Leucaena* at all supplementation levels. The total protein digestibility of M/LH (3 : 1) and M/LL (3 : 1) was comparable to both *Leucaena* hybrid and local. The protein digestibility of M/LH (1 : 1) and M/LL (1 : 1) were comparable but they were higher than M/LH (3 : 1) and M/LL (3 : 1) mixture.

Figure 1 shows the measured and predicted value of protein digestibility in the rumen from different mixture of mulberry and *Leucaena* at different level. The predicted values were calculated from the incubation of individual feeds which then extrapolated. It shows that the measured value in protein degradation of mulberry and *Leucaena* mixture in buffered rumen media was lower than the predicted value. The highest difference between measured and predicted protein degradation was observed at M/LH (3 : 1) (42%).

Figure 2 shows the measured and predicted values of protein digestibility in acid pepsin from the mixture of mulberry and *Leucaena*. It shows that the measured of protein digestibility was higher than the predicted values. The highest difference between measured and predicted values was in the M/LH (3 : 1) (65%).

The effect of *Leucaena* mixed with mulberry on VFA production is shown in Table 5. The mixture of mulberry and *Leucaena* significantly decreased total VFA compared to mulberry alone. However, the total VFA production was not significantly different between the mixture with different *Leucaena* varieties and ratios. Total VFA production of all mixtures was not significantly different to *Leucaena* variety alone except for M/LL (1 : 1) mixture.

There were no significant differences in the proportion of butyric acid among treatments. The exception was in the M/LH (3 : 1) mixture which had significantly higher value than the hybrid *Leucaena*. Mulberry had significantly higher proportion of iso valeric acid than other diets except for M/LH (3 : 1). However, the proportion of iso valeric acid of M/LH (3 : 1) was only significantly different to hybrid *Leucaena*. The proportion of Acetic acid, propionic acid and iso butyric acid was comparable (P>0.05) among treatments.

Table 3. Estimates of *in vitro* true dry matter digestibility (IVTDMD), fermentable substrate, volume gas production derived from 24 hours in vitro fermentation of 500 mg DM of the experimental diets

Treatment	Gas production (ml)	IVTDMD (%)	Substrate fermented (DM, mg)
Mulberry (M)	85.7^{a}	80.5^{a}	402.7^{a}
Leucaena Hybrid (LH)	59.5^{e}	75.8^{b}	378.9^{b}
Leucaena Local (LL)	55.8^{f}	67.3^{c}	336.4^{c}
M/LH (1 : 1)	74.3^{c}	77.5^{ab}	387.4^{ab}
M/LH (3 : 1)	67.6^{d}	68.8^{c}	344.0^{c}
M/LL (1 : 1)	76.9^{b}	73.7^{b}	368.3^{b}
M/LL (3 : 1)	75.6b^{c}	73.6^{b}	368.2^{b}
S.E.M.	0.78	1.46	0.23

Means with different superscript in the same column are significantly different (P<0.05)
S.E.M.: standard error means
M/LH (1 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 50 : 50%
M/LH (3 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 75 : 25%
M/LL (1 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 50 : 50%
M/LL (3 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 75 : 25%

Table 4. Estimates apparent protein digestibility of mulberry, Leucaena and mixed mulberry and Leucaena after incubation for 24 hours in rumen buffer media followed by acid pepsin digestion

Treatment	Microbial protein digestion (%)	Acid pepsin digestion (%)	Total protein digestion
Mulberry (M)	83.7^{a}	7.6^{d}	91.3^{a}
Leucaena Hybrid (LH)	13.6^{d}	55.9^{b}	69.4^{c}
Leucaena Local (LL)	15.7^{cd}	53.6^{b}	69.4^{c}
M/LH (1 : 1)	13.6^{d}	60.2^{a}	73.8^{b}
M/LH (3 : 1)	18.8^{bc}	48.8^{c}	67.6^{c}
M/LL (1 : 1)	21.0^{b}	55.4^{b}	76.4^{b}
M/LL (3 : 1)	21.7^{b}	46.5^{c}	68.2^{c}
S.E.M. it is better to put std for each value.	1.37	0.86	0.93

Means with different superscript in the same column are significantly different (P<0.05)
S.E.M. = standard error means
M/LH (1 : 1) = mulberry and *Leucaena leucocephala hybrid* mixture at ratio 50 : 50%
M/LH (3 : 1) = mulberry and *Leucaena leucocephala hybrid* mixture at ratio 75 : 25%
M/LL (1 : 1) = mulberry and *Leucaena leucocephala* local mixture at ratio 50 : 50%
M/LL (3 : 1) = mulberry and *Leucaena leucocephala* local mixture at ratio 75 : 25%

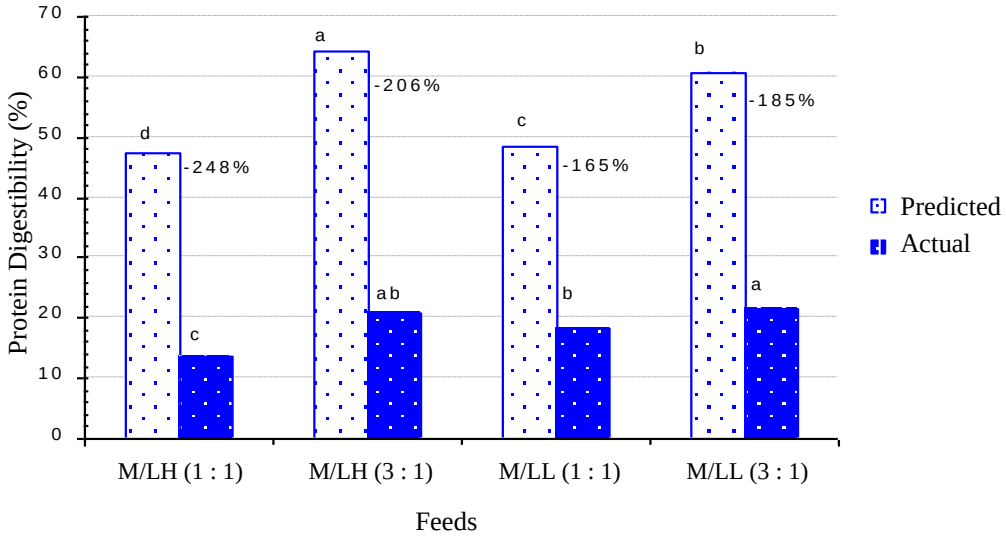

Figure 1. Protein degradability in rumen buffered media extrapolated from incubation of individual feed and measured by incubation of two feeds in different combination M/LH (1 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 50 : 50%; M/LH (3 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 75 : 25%; M/LL (1 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 50 : 50%; M/LL (3 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 75 : 25%).

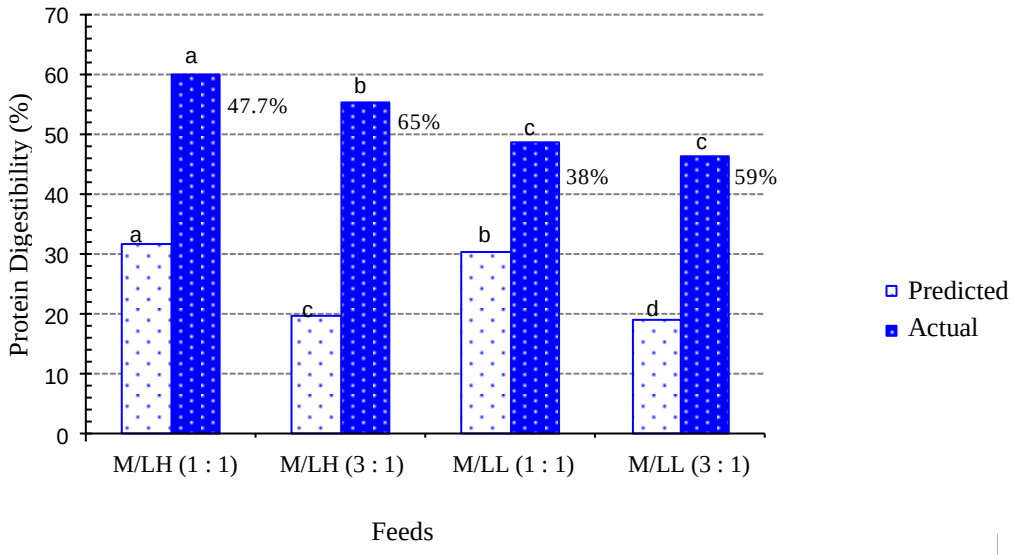

Figure 2. Protein digestibility in acid pepsin solution media extrapolated from incubation of individual feed and measured by incubation of two feeds in different combination M/LH (1 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 50 : 50%; M/LH (3 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 75 : 25%; M/LL (1 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 50 : 50%; M/LL (3 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 75 : 25%.

Discussion

In vitro digestibility

There is a good agreement between the magnitude of IVTOMD to the corresponding gas production. The high the IVTOMD value the higher is the gas production (Makkar 2003). However, in this study though gas production of mulberry was reduced when it was mixed with leucaena, but in mixture mulberry leucaena hybrid 50% (M/LH= 1 : 1) its IVTOMD was not significantly decreased (Table 3). Moreover, in Leucaena hybrid, though its gas production was significantly lower than other diets treatment, its IVTOMD was comparable to other diets except for mulberry. This indicates that the levels of tannin presented in the mixture diets did not limit the fermentability of the feeds. Getachew et al. (2000) and Guerrero et al. (2012) also reported that despite the lower gas production of tanniferous browses, its true digestibility was not affected by the presence of tannin. Similarly Norrapoke et al. (2014) also observed in rice straw diet supplemented by plant containing condensed tannin and saponin (mangosteen powder) did not reduce feed digestibility but decreased gas production. This condition was caused by the leaching of tannins from feed during fermentation, contributing to the DM loss but without contributing to the gas production or inhibition of cell soluble (Getachew et al. 1998; Makkar et al. 1997).

Further more, condensed tannin in the feed have more pronounced effect on protein degradability (Tiemann et al. 2008). However, Huang et al. (2010) reported that addition of various levels (2-5%) purified tannin from leucaena hybrid which was similar leucaena variety used in the current study to *Panicum maximum* grass substrate was able to reduce both *in vitro* DMD and total gas production, but the extent of reduction in gas production was higher than in digestibility. The gas decline by 19.68% when tannin was added at 2% and further decline to maximum 33.63% when the tannin addition was increased into 4% DM In this study though the levels of tannin addition (based on tannin content of leucaena) was higher (6.45% and 3.13% at leucaena hybrid mixture at 50% and 25% respectively the reduction of gas production was lower (13.3% and 21% respectively for mixture at 50 and 25%) the difference of gas production due to tannin addition in the current study with previous study reported by Huang et al. (2010) might be caused by the difference of tannin activity in extracted form (Huang et al. 2010) or in intact in the plant materials form such in the current study. Naumman et al. (2015) also reported that at different level of tannin content and different source of tannin in the diet did not affect total gas production. This indicated that effect of tannins on feed fermentation varied. Different tannin from different feedstuff have different inhibitory effect on rumen microbial activity which in turn affect on feed fermentation (Singh et al. 2005; Tiemann et al. 2008).

Table 5. Total VFA production and proportion of VFA of mulberry, Leucaena and their mixture after 24 h incubation

Treatment	Total VFA (mM)	Proportion of VFA (%)					
		C2	C3	C4	C5	C4i	C5i
Mulberry (M)	87.8^{a}	66.6	22.3	7.11^{ab}	1.23^{d}	1.04	1.92^{a}
Leucaena Hybrid (LH)	48.6^{c}	67.0	22.7	6.24^{b}	1.34^{cd}	0.95	1.12^{c}
Leucaena Local (LL)	37.3^{c}	67.0	22.0	6.47^{ab}	2.20^{a}	0.90	1.39^{bc}
M/LH (1 : 1)	58.3^{bc}	67.5	21.3	7.14^{ab}	1.59^{bcd}	0.97	1.51^{b}
M/LH (3 : 1)	47.6^{bc}	65.6	22.1	7.64^{a}	1.75^{bc}	1.15	1.74^{ab}
M/LL (1 : 1)	66.6^{b}	66.6	21.7	7.23^{ab}	1.84^{ab}	1.03	1.55^{b}
M/LL (3 : 1)	51.0^{bc}	68.2	20.9	7.03^{ab}	1.49^{bcd}	0.93	1.40^{bc}
S.E.M.	6.76	2.11	0.66	0.44	0.15	0.1	0.13

Means with different superscript in the same column are significantly different (P<0.05)
S.E.M. = standard error means
C2 = acetic acid
C3 = propionic acid
C4i = iso butyric acid
C4 = butyric acid
C5i = iso valeric acid
C5 = valeric acid
M/LH(1:1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 50 : 50%
M/LH (3 : 1): mulberry and *Leucaena leucocephala hybrid* mixture at ratio 75 : 25%
M/LL (1 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 50 : 50%
M/LL (3 : 1): mulberry and *Leucaena leucocephala* local mixture at ratio 75 : 25%

In vitro N digestibility

The mixture of mulberry with 50% hybrid *Leucaena* resulted in the lowest IVND than other treatments. When the level of the *Leucaena* decreased, the N degradability was increased by 50%, indicating that the level of tannin content in *Leucaena* affect the N degradability in the rumen (Table 4). Huang et al. (2010) reported addition of 2% purified tannin from Leucaea hybrid reduce degradability by 17.65% of protein *Panicum maximum* grass, further increased of tannin addition up to 4% did not resulted in further decreased of the protein substrate. Anti nutritional effect of tannin is exerted through reduction of feed protein digestion and a depression of proteolytic enzyme activities (Jones et al. 1994). A reduction in crude protein degradability is the consequence of condensed tannin (CT) binding to dietary constituents and to bacteria. In addition, the CT can reduce attachment of bacteria to plant particle (McAllister et al. 1994). Getachew et al. (2000) reported that IVDN increased when PEG (tannin binding agent) was added to the tannin-rich browse. The increase of the extent of N digestibility by rumen microbial due to PEG addition indicates the amount of protein protected by tannin from microbial degradation. In the present study, the protein degradability of mulberry-*Leucaena* mixture was reduced compared to mulberry alone. The decrease of protein degradability could be due to the effect of tannin from *Leucaena*. Previously Williams et al. (2011) reported that total mixed ration of sainfonin hay based-diet contained 3.8% tannin, rumen ammonia nitrogen content of the diet was lower compared to alfalfa hay based-diet with low tannin content (0.2%), the lower rumen ammonia nitrogen as indication of protein protection by tannin in the rumen. The *in vitro* protein degradability in M/LH (1 : 1) mulbery-*Leucaena* mixture was lower than other mixtures (Table 3). It shows that hybrid *Leucaena* has higher effect in decreasing protein degradability than *Leucaena* local (M/LL). The Leucaena varieties used in this study was similar tree reported by Khamseekhiew (2005) and Huang et al. (2010). Khamseekhiew (2005) reported that hybrid *Leucaena* had higher CT content than *Leucaena* local, which caused the lower N degradability in the former. In addition, CT in hybrid *Leucaena* had stronger binding affinity than *Leucaena* local (Huang et al. 2010). The CT which have stronger affinity and have higher molecular weight have higher ptotein binding affinity than those with lower molecular weight (Saminathan et al. 2015). Similar results was also reported by Kariuki & Norton (2008) that within Leucaena genus has different ability in protecting protein. Moreover Cortes et al. (2009) also observed that effect of tannin in protecting protein beside affected by source of tannin also by ratio of tannin added.

The protein degradability of mulberry alone was higher, however when it was mixed with leucaena the protein degradability was reduced though the protein content of this mixture was higher than mulberry (Table 2). The measured protein degradability was lower than the predicted value, indicating that there was an associative effect between mulberry and leucaena when it was incubated together. According Getachew et al. (2005) combination of different feed ingredients incubated together with condition that one feed is not independent to other feed will results in associative effect which can be detected when the response of the combination is not linear. This associative effect caused by the presence of feed ingredients stimulate rumen fermentation and consequently its affect the digestibility of other feed ingredients. Through the positive associative effect, the present study shows that protein digestibility in acid pepsin was higher in the measured values than in the predicted value. Protein availability for digestion in the intestines (acid pepsin) increased from only 8% in mulberry to 82% in M/LH (1 : 1) mulberry-*Leucaena* mixture rate. Although the total protein digestibility decreased from 91.3% in mulberry to 73.8% in M/LH (1 : 1) or 76.4% in M/LL (1 : 1), the protein digestion was available for the absorption in the intestine. The acid pepsin digestible protein could be regarded as a potential rumen by pass protein. McSweeney et al. (1999) reported that in browse (Lucerne) containing no tannin, accumulation of NH_3-N has strong correlation with N degradation. On the other hand, in tannin containing shrub legumes had lower potential N digestibilities in the rumen. However, a large portion is available following the acid pepsin digestion compared to Lucerne, this was due to protein tannin complex would be dissociated post-ruminally. McLeod (1974) as referred by Norton & Ahn (1997) observed that tannin bind protein in the rumen at pH 5.8-6.8 and the linkages are strongly dependent on pH. The linkage being stable at pH 3.5-7.0, but this tannin-protein complexes should be dissociated in the abomasum (pH 2.5-3.5) and in small intestine (pH 7.5-8.5). Therefore protecting protein from digestion in the rumen is the advantage of the presence protein-tannin complex in the diet thereby increasing total supply of feed protein for absorption (Makkar 2003).

VFA production

The higher total VFA production in mulberry than other diets (Table 5) was a results from the high IVTDMD of mulberry (Table 3) this due to VFA is one of the products beside gases and microbial cell from carbohydrates fermentation (Makkar 2000). Mixing leucaena to mulberry resulted in the decreased of total VFA production. The decrease of total VFA was also reflected in lower IVTDMD and gas production of

mulberry-leucaena mixed diets (Table 3). The decrease of fermentability these diets could be caused by the present of tannin from leucaena. Tannin decrease attachment of fibre digesting microbes to feed particles (McAllister et al. 1994) therefore decrease rate of digestion which in turn decreased VFA production. This is consistent with the previous study that showed the tannin content in tree browse reduced the gas and total VFA production (Getachew et al. 2002; Khamseekhiew 2005). However, the molar proportion of acetic and propionic, and iso butyric acid was not affected by treatment. On the other hand, the molar proportion of iso-valeric in mulberry-leucaena mixed diet was significantly decreased compared to mulberry. The decreased of iso-valeric which mostly from deamination of some amino acid (Copani et al. 2015) in mixed mulberry leucaena suggest that protein of mulberry was protected by condensed tannin from leucaena. Simlar result was also reported by Copani et al. (2015) in mixture of Timothy grass and Sainfonin, and by Niderkorn et al. (2012) in mixture of cocksfood and sainfonin.

CONCLUSION

Supplementation of *Leucaena* to mulberry decreased *in vitro* true dry matter digestibility (IVDMD) and decreased protein digestibility in the buffered rumen fluid media. *Leucaena* supplementation could increase protein digestibility in acid pepsin incubation as indicator of protein availability in intestine. Hybrid *Leucaena* supplementation to mulberry at ratio 1 : 1 was the most effective level to decrease protein digestion in the rumen and to increase protein digestibility in acid pepsin incubation.

REFERENCES

Anbarasu C, Dutta N, Sharma K, Rawat M. 2004. Response of goats to partial replacement of dietary protein by a leaf meal mixture containing *Leucaena leucocephala, Morus alba* and *Tectona grandis*. Small Rum Res. 51:47-56.

[AOAC] Association of Official Analytical Chemist. 2000. Official method of analysis. 17th ed. Washington DC (USA): Association of Official Analytical Chemist.

Barry TN, McNabb WC. 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. Br J Nutr. 81:263-272.

Williams CM, Eun JS, MacAdam JW, Young AJ, Fellner V, Min BR. 2011. Effects of forage legumes containing condensed tannins on methane and ammonia production in continuous cultures of mixed ruminal microorganisms. Anim Feed Sci Technol. 166-167:364-372.

Camacho LM, Rojo R, Salem AZM, Mendoza GD, López D, Tinoco JL, Albarrán B, Monta˜nez-Valdez OD. 2010. *In vitro* ruminal fermentation kinetics and energy utilization of three Mexican tree fodder species during the rainy and dry period. Anim Feed Sci Technol. 160:110-120.

Copani G, Ginane C, Le Morvan A, Niderkorn V. 2015. Patterns of *in vitro* rumen fermentation of silage mixtures including sainfoin and red clover as bioactive legumes. Anim Feed Sci Technol. 208:220-224.

Cortés JE, Moreno B, Pabón ML, Avila P, Kreuzer M, Hesse HD, Carulla JE. 2009. Effects of purified condensed tanins extracted from Calliandra, Flemingia and Leucaena on ruminal and postruminal degradation of soybean meal as estimated *in vitro*. Anim Feed Sci Technol. 151:194-204.

Gemeda BS, Hassen A. 2015. Effect of tannin and species variation on *In vitro* digestibility, gas, and methane production of tropical browse plants. Asian-Australas J Anim Sci. 28:188-199.

Getachew G, Pittroff W, Putnam DH, Dandekar A, Goyal S, DePeters EJ. 2008. The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on *in vitro* rumen fermentation and microbial protein synthesis. Anim Feed Sci Technol. 140:444-461.

Getachew G., Blummel M, Makkar HPS, Becker K. 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds: a review. Anim Feed Sci Technol. 72:261-281.

Getachew G, DePeters EJ, Robinson PH, Fadel JG. 2005. Use of an *in vitro* rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. Anim Feed Sci Technol. 123-124:547-559.

Getachew G, Makkar HPS, Becker K. 2000. Effect of polyethylene glycol on *in vitro* degradability of nitrogen and microbial protein synthesis from tannin-rich browse and herbaceous legumes. Br J Nutr. 84:73-83.

Getachew G, Makkar HPS, Becker K. 2002. Tropical browses: contents of phenolic compound and stoichiometric relationship between short chain fatty acid and *in vitro* gas production. J Agr Sci Camb. 139:341-352.

Huang XD, Liang JB, Tan HY, Yahya R, Khamseekhiew B, Ho YW. 2010. Molecular weight and protein binding affinity of Leucaena condensed tannins and their effects on *in vitro* fermentation parameters. Anim Feed Sci Technol. 159:81-87.

Huyen NT, Wanapat M, Navanukraw C. 2012. Effect of Mulberry leaf pellet (MUP) supplementation on rumen fermentation and nutrient digestibility in beef cattle fed on rice straw-based diets. Anim Feed Sci Technol. 175:8-15.

Jones GA, McAllister TA, Mair AD, Cheng KJ. 1994. Effect of sainfonin (*Onobrychis viciaefolia* Scop) condensed tannin on growth and proteolysis by four strains of

ruminal bacteria. Appl Environ Microbiol. 60:1374-1378.

Kariuki IW, Norton BW, Kariuki. 2008. The digestion of dietary protein bound by condensed tannins in the gastro-intestinal tract of sheep. Anim Feed Sci Technol. 142:197-209.

Khamseekhiew B. 2006. Characteristics and protein binding affinity of condensed tannins in leucaena species (Thesis). [Malaysia (Malaysia)]: University Putra Malaysia.

Guerrero M, Cerrillo-Soto MA, Ramírez RG, Salem AZM, González H, Juárez-Reyes AS. 2012. Influence of polyethylene glycol on *in vitro* gas production profiles and microbial protein synthesis of some shrub species. Anim Feed Sci Technol. 176:32-39.

Makkar HPS. 2003. Effects and fate of tannin in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds: Review. Small Rumin Res. 49:241-256.

Makkar HPS, Blummel M, Becker K. 1997. *In vitro* rumen apparent and true digestibilities of tannin-rich forages. Anim Feed Sci Technol. 67:245-251.

McAllister TA, Bae HD, Jones GA, Cheng KJ. 1994. Microbial attachment and feed digestion in the rumen. J Anim Sci. 72:3004-3018.

McSweeney CS, Palmer B, Bunch R, Krause DO. 1999. *In vitro* quality assessment of tannin-containing tropical shrub legumes: protein and fibre digestion. Anim Feed Sci Technol. 82:227-241.

McSweeney CS, Palmer B, McNeill DM, Krause DO. 2001. Microbial interaction with tannins: nutritional consequences for ruminants. Anim Feed Sci Technol. 91:83-93.

Naumann HD, Lambert BD, Armstrong SA, Fonseca MA, Tedeschi LO, Muir JP. 2015. Effect of replacing alfalfa with panicled-tick clover or sericea lespedeza in corn-alfalfa-based substrates on *in vitro* ruminal methane production. J Dairy Sci. 98:3980-3987.

Naumann HD, Hagerman AE, Lambert BD, Muir JP, Tedeschi LO, Kothmann MM. 2014. Molecular weight and protein-precipitating ability of condensed tannins from warm-season perennial legumes. J Plant Interactions. 9:212-219. DOI: 10.1080/17429145.2013. 811547.

Niderkorn V, Mueller-Harvey I, Le Morvan A, Aufrère J. 2012. Synergistic effects of mixing cocksfoot and sainfoin on *in vitro* rumen fermentation Roleof condensed tannins. Anim Feed Sci Technol. 178:48-56.

Norrapoke T, Wanapat M, Foiklang S. 2014. Influence of tropical plant sources containing plant secondary compound on rumen fermentation using *in vitro* gas fermentation technique. Indian J Anim Sci. 84:1004-1010.

Norton BW, Ahn JH. 1997. A comparison of fresh and dried *Calliandra calothyrsus* supplements for sheep given a basal diet of barley straw. J Agr Sci Camb. 129:485-494.

Palmer B, Jones RJ. 2000. The effect of PEG addition on dry matter and nitrogen digestibility of *Calliandra calothyrsus* and *Leucaena leucocephala* leaf. Anim Feed Sci Technol. 85:259-268.

Saddul D. 2005. Evaluation and utilization of *Morus alba* (mulberry) as a protein supplement for ruminants (Thesis). [Malaysia (Malaysia)]: University Putra Malaysia.

Saddul D, Jelan ZA, Liang JB, Halim RA. 2004. Effect of high drying temperatures on protein fraction and *in vitro* gas production mulberry foliage. Proceeding 11th Animal Science Congress. The Asian-Australasian Association of Animal Production Societies. Kuala Lumpur (Malaysia): Malaysian Society of Animal Production. p. 402-404.

Saminathan M, Tan HY, Sieo CC, Abdullah N, Wong CMVL, Abdulmalek E, Ho YW. 2014. Polymerization degrees, molecular weights and protein-binding affinities of condensed tannin fractions from a *Leucaena leucocephala* hybrid. Molecules. 19:7990-8010. DOI: 10.3390/molecules19067990

SAS. 2002. SAS/STAT user's guide (Release 9.1). North Carolina (USA): SAS Institute.

Singh B, Sahoo B, Sharma R, Bhat TK. 2005. Effect of polyethylene glycol on gas production parameters and nitrogen disappearance of some tree forages. Anim Feed Sci Technol. 123-124:351-364.

Tiemann TT, Avila P, Ram´ırez G, Lascano CE, Kreuzer M, Hess HD. 2008. *In vitro* ruminal fermentation of tanniniferous tropical plants: Plant-specific tannin effects and counteracting efficiency of PEG. Anim Feed Sci Technol. 146:222-241.

Tilley JM, Terry RA. 1963. A two-stage technique for the *in vitro* digestion of forage crops. Br J Nutr. 18:104-111.

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci. 74:3583-3593.

Yulistiani D, Jelan ZA, Liang JB, Yaakub H, Abdullah N. 2015. Effects of supplementation of mulberry (*Morus alba*) foliage and urea-rice bran as fermentable energy and protein sources in sheep fed urea-treated rice straw based diet. Asian Australas J Anim Sci. 28:494-501.

Yulistiani D, Jelan ZA, Liang JB. 2008. Degradability of mulberry (*Morus alba*) and rice bran in the rumen of sheep fed different diets. Indones J Anim Vet Sci. 13:264-272.

Anaerobic Fermentation Effectively Reduces Concentration of Total Tannins in *Chromolaenan odorata*

Mullik YM[1,2], Ridla M[1], Prihantoro I[1], Mullik ML[2]

[1]*Faculty of Animal Science, Graduate School, Bogor Agricultural University, Indonesia*
[2]*Faculty of Animal Science, University of Nusa Cendana, Indonesia*
E-mail: martin_kpg@yahoo.com.au

(received 22-01-2016; revised 24-03-2016; accepted 29-03-2016)

ABSTRAK

Mullik YM, Ridla M, Prihantoro I, Mullik ML. 2016. Fermentasi anaerobik efektif menurunkan konsentrasi total tanin pada tumbuhan semak bunga putih (*Chromolaena odorata*). JITV 21(1): 19-25. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1301

Chromolaena odorata merupakan sumber pakan alternatif potensial, namum penggunaannya terkendala oleh kandungan berbagai senyawa metabolik sekunder dalam jaringan tumbuhan ini. Salah satu kelompok senyawa tersebut adalah tannin. Penelitian ini bertujuan untuk mengevaluasi pengaruh berbagai metode perlakuan awal terhadap total konsentrasi tannin dan daya cerna bahan kering dan bahan organik yang diukur secara *in vitro* dan konsentrasi produk fermentasi rumen. Rancangan acak lengkap (8 x 3) digunakan untuk menguji perbedaan 8 jenis perlakuan yaitu daun *chromolaena* segar sebagai kontrol (*Fresh*), dijemur selama 3 x 24 jam (*Sun-dried*), dikeringkan pada suhu 60°C selama 24 jam (*Oven-dried*), direbus dalam air selama 5 menit (*Boiled*), direndam dalam air biasa selama 4 jam (*RenWater*), direndam dalam NaOH selama 4 jam (RenNaOH), direndam dalam HCl selam 4 jam (RenHCl), atau difermentasi secara anaerobik selama 21 hari (*Fermented*). Parameter yang diukur adalah konsentrasi tannin total dan kandungan nutrisi dalam setiap bahan yang mendapat perlakuan tertentu. Hasil penelitian menunjukkan bahwa perlakukan Jemur, Rebus, RenAir, dan fermentasi secara nyata menurunkan total tannin sehingga besar 43% hingga 62% dibandimg kontrol. Penurunan terbesar (62%) ditunjukkan oleh perlakukan Fermentasi. Sebaliknya, penggunaan panas tinggi (dioven) atau bahan kimia (HCL dan NaOH) tidak nyata menurunkan konsentrasi tannin. Kandungan protein kasar meningkat sebesar 60% dan serat kasar menurun sebesar 32% pada perlakuan fermentasi dibanding kontrol. Disimpulkan bahwa metode fermentasi anaerobic dapat digunakan sebagai strategi efektif untuk menurunkan konsentrasi tannin dalam tumbuhan semak bunga putih (*Chromolaena odorata*) tanpa mengurangi nilai nutrisinya sebagai bahan pakan.

Kata Kunci: *Chromolaena odorata*, Tannin, Daya Cerna, NH_3, VFA

ABSTRACT

Mullik YM, Ridla M, Prihantoro I, Mullik ML. 2016. Anaerobic fermentation effectively reduces concentration of total tannins in *Chromolaena odorata*. JITV 21(1): 19-25. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1301

Chromolaena odorata is a potential feed source but its usage is hampered by presence of various secondary metabolic compounds in plant's tissues. One group of them is tannin. This experiment was aimed to evaluate various pre-treatment methods on total tannin concentration and in vitro digestibility of dry- and organic-matter. An 8 x 3 completely randomized experimental design was employed to test 8 different treatments. The treatments were: Fresh = freshly-chopped chromolaena leaves as control, Sun-dried = sun-dried (3x 24 hours) chromolaena leaves, Oven-dried = oven-dried (60ºC for 24 hours) chromolaena leaves, Boiled = water-boiled (5 minutes) chromolaena leaves, RenWater = water-soaked (4 hours) chromolaena leaves, RenNaOH = NaOH-soaked (4 hours) chromolaena leaves, RenHCl = HCl-soaked (4 hours) chromolaena leaves, and Fermented = anaerobically-fermented (21 days) chromolaena leaves. Parameters measured were concentration of total tannins and nutrient content. The results showed that application of low heat (Sun-dried), hot water (Boiled), water soaking (RenWater), or unaeobic fermentation technique significantly reduced total tannin by 43% into 62% compared to control. The highest suppression (62%) was achieved by Fermented treatment. In the contrary, medium heat application (oven-dried at 60°C) or chemicaltreatments (HCl or NaOH) had no effect. Protein content of chromolaena was improved by 60% and crude fiber was reduced by 32% in Fermentaed treatment compared to the control. It could be concluded that anaerobic fermentation can be used an effective strategy to reduce tannin concentration in *Chromolaena odorata* without affecting its feeding value.

Key Words: *Chromolaena odorata*, Tannins, Digestibility, NH_3, VFA

INTRODUCTION

Siam weed (*Chromolaena odorata*) is a pasture weed for Eastern Indonesia rangelands which have extremely high biomass production (up to 70 ton DM/ha/year) with crude protein content of about 21-36% (Mullik 2002) yet contains various anti-nutrient agents in the form of secondary methabolic compounds. Some of these secondary methabolic properties are tannins, anti-trypsine, haemaglutinnine, saponine,

oxalate, pitate acid, alkaloids, steroids, terpoids, and flavonoids (Akinmoladun et al. 2010; Onkaramurthy et al. 2013). The presence of these compounds lower palatability index of *C. odorata* (Hai et al. 2012) due to strong mint odour and relatively bitter taste. Ruminants rarely consume *C. odorata* in a fresh form. Therefore, pre-treatment is required to overcome these nutritional and intake limitations. The pre-treatments should be directed to reduce or eliminate anti-nutrient compouds but maintain nutrient quality and safe for animals and environment.

Tannin goup is a dominant anti-nutrient compound in *C. odorata* (Onkaramurthy et al. 2013). Reducing or eliminating tannins is likely to increase palatability and feeding value of this plant. Provision of feedstuff with high tannin content as single diet to livestock could surpress feed intake, palatability, daily weight gain (Wina 2010), protein degradation in rumen due to tannin-protein binding effects (Patra & Saxena 2011), and toxic for rumen microbes (Bhatta et al. 2009). Tannins also have the potency to disrupt digestive tract function (Makkar 2003) due to an inhibition in the activities of digestive enzymes such as proteases, lipases, and glicocidases (Hagerman 1992).

Physical, chemical and biological treatments are existing pre-treatment methods used to reduce or eliminate tannins in feedstuff (Roger et al. 2015). Physical treatments (chopping, milling, pelleting), chemical treatments (heating, soaking in water or acid or alkali solution), and biological treatment (microbial fermentation) have been adopted worldwide in feed processing. Thus, they could be used to treat *C. odorata* to reduce its anti-nutrient properties. However, various researchers have shown that response of tannins to treatment is not consistant among feed sources. As an example, Hue et al. (2010) reported that withering and drying significantly reduce tannin concentration in cassava leaves, yet Wina et al. (2000) found an incrase in tannins in aerobically-dried *Calliandra calothyrsus* leaves. This inconsistency could be related to high variability in chemical structures of tannins among plants and plant materials (Patra & Saxena 2011; Gemede & Hassen 2015).

Due to the inconsistency in tannin response to processing, it is not clear, what is the effective pre-treatment method to reduce tannin concentration in *C. odorata*. Two latest studies (Mullik et al. 2014; Bira et al. 2015) showed that a serial pre-treatments (sun-drying, milling, and pelleting) still not able to guarantee that *C. odorata* can be used as a safe feed source for cattle. These researchers (Mullik et al. 2014; Bira et al. 2015) found that total feed intake, digestibility and rumen fermentation begins to decrease as inclusion of chromolaena meal in the diet increased from 30% to 40%. Based on these findings, the present study was designed to test effects of sun-drying, oven-drying, water-boiling, soaking in water or HCl and NaOH solution, and fermentation on total tannins and nutrient content of *C. odorata*.

MATERIALS AND METHODS

This study was carriedout in August-December 2014. Tannin evaluation and analysis of nutrition content of *C. odorata* was conducted at Animal Feed Technology Laboratory (ITP) of Faculty of Animal Science, Bogor Agricultural University. *C. odorata* leaves used in this study were obtained from Kupang, East Nusa Tenggara. The leaves were harvested by pruning the plants at a height of ±50 cm from the ground. The leaves were then separated from the rod and assigned to the treatments.

This study used 8 treatmets which were arranged in a 8 x 3 Completely Randomized Design. The treatments were:

Fresh = Fresh *C. odorata* as control
Sun-dried = *C. odorata* was sun-dried for 3 x 24 hours
Oven-dried = *C. odorata* was 60^{o}C oven-dried for 24 hours
Boiled = *C. odorata* was boiled for 5 minuts
RenWater = *C. odorata* was soaked in the water for 4 hours
RenNaOH = *C. odorata* was soaked in NaOH 0.1N 10% for 4 hours
RenHCl = *C. odorata* was soaked in HCl 0.1 N 10% for 4 hours
Fermented = *C. odorata* was fermented without additive ingredient for 21 days

Sampling and handling procedures

For the first treatment (Fresh), *C. odorata* leaves were chopped as fine as possible and then used for analysis. For the sun-dried treatment, *C. odorata* leaves were spread on top of a tarp, dried under sun light for 3 days, finely grounded and then analyzed. For oven-dried treatment, *C. odorata* leaves were placed in a tray and fed into oven at 60°C for 24 hours. Dried materials was then grounded, and analyzed. For boiled treatment, *C. odorata* leaves were boiled in water (with a ratio of 100 g leaves/150 mL water) for 5 minutes using medium heat. The boiled materials was then filtered, wind-dried, finely chopped, and then analyzed for chemical composition. For the RenWater treatment, *C. odorata* leaves was soaked in plain water (a ratio of 200 g leaves/1000 mL water) at room temperature for 4 hours. For RenNaOH and RenHCl treatment procedures were same with the RenWater, but the solution used was 0.1N NaOH or 0.1N HCl (Ratio: 200 g leaves/1000 mL of 10% NaOH or 10% HCl). After soaking, the samples

were washed with fresh water to reduce negative effect of NaOH and HCl, filtered, wind-dried, finely ground, and analiyzed. For fermentated treatment, fresh leaves were chopped, arranged into 1 litter jar, densified, air-tight sealed, and fermented for 21 days. After fermentation, the feedstuff was unpacked, wind-dried, then finely ground prior laboratory analyzes for chemical composition. All chemical analyzes were done at Animal Feed Technology Laboratory (ITP) of Faculty of Animal Science, Bogor Agricultural University.

Parameters and measurements

Total tannins content

Quantification of total tannins was done by analizing samples from each treatment. Sample (1.2 g) was extracted with aquades in 100 mL volumetric flask for 4 hours at room temperature, filtered using whatman paper number 40. About 10 mL supernatant was pippeted into 500 mL volumetric flask where 10 mL *indigo carmine* solution and 300 mL aquades were then added. This solution was then destilled using 0.1N $KMnO_4$ until the colour of the solution change from blue to green. The titration process continued until the colour of the solution turned into golden yellow. Standar solution of *indigo carmine* was made by dissolving 3 g of *indigo carmine* in 250 mL hot aquades and then cooled. After colling, 25 mL H_2SO_4 was added and diluted into 500 mL aquades. The mixture was then allowed to cool down befored filtered. The blank standard was made of 10 mL *indigo carmine* solution and 300 mL aquades.

Total tannin concentration was obtained by using titrimetric method according to Atanassova & Christova-Bagdassarian (2009) modified from The International Pharmacopoeia (2003) and AOAC (1965).

$$T\,(\%) = \frac{(V - Vo)x\ 0.004157\ x\ 250\ x\ 100}{g\ x\ 25}$$

where:

V = volume of 0.1N $KMnO_4$ for sample titration (mL)

Vo = volume of 0.1N $KMnO_4$ for blanco sample titration (ml)

0.004157= tannin was equivalent in 1 mL 0.1N $KMnO_4$

G = sample mass used in the analysis (g)

250 = volume of volumetric flask (mL)

25 = volume of *Indigo carmine* (mL)

100 = percentage (%)

Nutrition content

Nutrition content determination in each sample was derived by chemical analysis in a dry matter basis (DM). The nutrients were ash, crude protein (CP), crude fat (CFat), crude Fiber (CF). The method employed was AOAC (2005). The results were used to compute nutrient content in the basis of g/kg DM or percentage (%).

Data analysis

Data were analyzed by general linear model for Completely Randomized Design with configuration of 8 treatments and 3 replicates. Treatment differences was set at α value of 0.05%. Data were analysid using SPSS version 23. Normalization of data was performed using transformation technique.

RESULT AND DISCUSSION

Total tannins

Total tannin concentration (Table 1) showed a decline trend for all treatments. The highest reduction (62%) was obtained in Fermentated treatment (0.94%) compared with control (2.17%). A medium decline in tannin concentration (43-53%) was shown by sun-drying (1.17%), water boiling (1.40%), and water soaking (2.31%). Lowest respons (4-9%) was detected in Oven-dried (2.23%), RenNaOH (2.31%), and RenHCl (2.36%). Total tannin concentration detected in the current experiment fit to the range of 1.3-17.2% reported by Gemeda & Hassen (2015) in various tropical shrub forages in South Africa.

Table 1. Total tannins of *Chromolaena odorata* subjected to various pre-treatment methods

Treatment	Total tannins (% DM)*
Fresh	2.47[b]
Sun-dried	1.17[a]
Oven-dried	2.23[b]
Boiled	1,40[a]
RenWater	1.31[a]
RenNaOH	2.31[b]
RenHCl	2.36[b]
Fermentated	0.94[a]
SEM	0.033
P-value	<0.001

*Different supercript in the same column shows significant difference at alfa 5%

Analysis variance showed that there were 4 treatments which significantly reduced tannin concentration by 43-62% compared with control. The treatments were Fermented, Boiled, RenWater, and

Sun-dried. Other treatments (Oven-dried, RenNaOH, and RenHCl) had no significant effect (P>0.05) compared with control. These results are in line with Roger et al. (2005) who showed that fermentation reduces total tannin concentration. Conclusive explanation about treatment effects on tannin concentration could not be established in the present experiment since types of tannins were not characterized or have not been published elsewere. However, reduction in tannin concentration in Fermented treatment could be the effect of chemical activities from enzymes produced by various fermentative microbes. These enzymes might damage tannin-enzyme and protein-tannin complex to release protein from tannin in the residual solution (Taylor & Duodu 2014). Furthermore, there was a possibility of continuing hydrolysis of soluble tannins by microbial enzymes to form other compounds during anaerobic fermentation.

A siginificant decreased in tannin concentration for water-boiled and water-soaked treatments could be related to dissolution of soluble tannin compounds (*gallotannin* goup) in the water. Unknown tannin composition (types of tannins) in the present study results in unknown proportion of soluble tannins compared with other three groups (*ellagitannins, complex tannins dan condensed tannins*). Dissolution of tannins in the water in the current study could be possible since most gallotannins have polyol residues derived from D-glucose (Khanbabaee & Van Ree 2001). Khanbabaee & Van Ree (2001) reported that gallotonnins type *2,3,4,6-tetra-O-galloyl-D-glucopyranose* and *1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose* are intermediary key compounds which have a crucial role in biosythesis of almost all phenolic compounds in plants. Available water would be a good medium for chemical reactions related to tannin hydrolysis.

Low heat application (sun-dried) also significantly reduced tannin concentration by about 53% in *C. odorata*. This is clearly different from medium heat application (oven-dried at 60°C) which showed an insignificant effect (only 9%) compared with control. Lesser tannin reduction (3.4%) was also reported by Rakić et al. (2004) who oven-drying oak skin at 60°C temperature. Djordjevic (1995) proposed that important reactions occur due to heating are hydrolysis, oxidation, polymerization, and interaction of composition and decomposition processes.

Different response of tannins to heat application could be explained by using statement of Hagerman (2002) that tannins reactions is highly affected by temperature. High temperature stimulate condensation reaction of tannins to form complex bond with other compounds. Slow drying at a low temperature, such as in Sun-dried treatment, might allow hydrolysis and decomposition of tannins (Makkar & Becker 1996). As tannins have hydrophobic bonds, it will form a strong ionic bond at high temperature (Haslam 1989). This might be the explanation to the insignificant reduction in Ovened-dried treatment.

No different in NaOH or HCl treatmet with control showed that tannins are less reactive to strong acid or basic solution. Lack of tannin response to strong acid (2M HCl) and base (2M NaOH) was also reported by Osawa & Walsh (1993) for tanic acid. This might related to ionization in hydroxyl phenol group in tannins (Hagerman 2002) hence protecting it from hydrolysiss processes.

Nutrition content

The effects of various treatments on nutrient content of *C. odorata* is shown in Table 2. There are three essential nutrient variables determining biological value of *C. odonata* which were affected by treatment i.e organic matter, crude protein, and crude fiber. Data in Table 2 shows that the highest organic matter was obtained in RenHCl treatment (922 g/kg DM) and the lowest one was in Fermented treatment (879 g/kg DM). Organic matter usually has high hydrogen thus increase opportunity for tannins to bind to other organic materials such as cellulose and hemicellulose (McSweeney et al. 2001). Theoritically, soaking of feed materials in acid solution, such as HCl, could cause hydrolysis of complex chemical bonds in organic compunds including tannin-protein or tannin-other organic materials. However, acid or basic affect in the present study was very low compared with other treatments. Data in Table 1 showed that HCl ability to reduce tannins in *C. odorata* was much lower than Fermented treatment. This possibly related to mode of hydrolysis between the two treatments. For RenHcl treatment, the only mode of hydrolysis was by acid effect since most of fermentative microbes might have died in strong acid solution. In contrast, hydrolysis processes in Fermented treatment is mostly done by a variety of fermentative microbes. Released organic matter in HCl treatment might not undergo further decomposition. In the Fremented treatment, even though it has a high ability to reduce tannins causing high amount of organic matter released from the complex, this organic matter might undergo further hydrolysis by microbe during fermentation hence reduces its proportion in the residu. In such a condition, addition of soluble carbohydrates (SCs) is trongly recommended. The SCs are important in fermentation processes as it will improve nutrient value and reduce

Table 2. Nutrient content of *C. odorata* due to treatments

Treatment	Nutrient content					
	Dry material	Organic material	Crude protein	Crude fat	Crude fiber	BETN
	---------- g/kg DM ----------					
Fresh	266[b]	908[c]	174[f]	30[e]	127[c]	578[a]
Sun-dried	839[a]	866[f]	258[b]	48[d]	108[b]	452[d]
Oven-dried	863[a]	888[d]	250[c]	67[c]	99[b]	471[bc]
Boiled	245[b]	914[b]	196[e]	106[a]	135[c]	477[b]
RenWater	271[b]	914[b]	228[c]	109[a]	145[d]	433[e]
RenNaOH	247[b]	867[f]	199[de]	62[c]	130[c]	474[b]
RenHCl	183[c]	922[a]	202[d]	107[a]	151[d]	462[cd]
Fermented	243[b]	879[e]	290[a]	75[b]	86[a]	429[e]
SEM	0.002	0.002	0.012	0.006	0.006	0.002
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

*Different supercrpit in the same column showssignificant different at alfa value of 5%

dry material losses (McDonald et al. 1991). The strategy is very crucial in silage making particularly for feedstuff with low C : N ratio.

The highest crude protein content (290 g/kg DM) was shown in Fermented treament and the lowest (174 g/kg DM) was in control (Fresh). Data in Table 2 shows that all treatments increased protein content above control. This might be explained by data in Table 1 that all treatments were able to decrease tannin concentration hence an increase in tannin free proteins. Fermentation increase crude protein of *C. odorata* due to its capability disrupt tannin-protein complex. Besides, chemical activity of fermentative microbes and lysis of tennin-enzyme and tannin-protein complexes (Taylor & Duodu 2014). It is presumed that an increase in protein content in the Fermented treatment is likely due to contribution of microbial proteins synthesized during fermentation process. Crude protein content in water-boiled treatment (196 g/kg DM) was higher than control but lower than other treatments. This possibly related to dissolution of tannin-protein complex. Heat from boiling likely to cause denaturation of protein hence releases proteins from protein-tannin linkage in *C. odorata*.

Lowest crude fiber (86 g/kg DM) was found in fermented treatment and the highest (145 g/kg DM) content was in RenWater treatment. A decline of crude fibre in Fermented ttreatment likely to be caused by fiber-digested bacterial activities during fermentation. Among cellulotic species, there are fiber digested bacteria to diegst cellulose, hemicellulose, and starch (Kana-Hau et al. 2005). Water soaking (RenWater) had a very poor capability to reduce crude fiber content. Fiber consists of cellulose, hemicellulose, and lignine. Usualy, cellulose and hemicellulose would be degraded with the help of cellulose and hemicellulose enzymes, whereas water has no direct effect on crude fiber degradtion.

Crude fat of fresh *C. odorata* was 30 g/kg DM and tended to increase after treatment. Highest fat content detected in RenWater treatment (109 g/kg DM). This increase might be caused by a significant loss in non-fat compounds, particularly carhohydrates due to fermentation and other processes. Reduction in non-fat organic compounds will automatically increase proportion of crude fat in feed materials.

BETN consist of highly digestible carbohydrate compounds. BETN content of fresh *C. odorata* was 578 g/kg DM. Lowest BETN content (429 g/kg DM) was in the Fermented treatment. BETN content in fresh *C. odorata* was very high since all soluble carbohydrates have not been degraded, whereas in the Fermented treatment, soluble carbohydrates have been degraded by microbes during the fermentation processes. Provision of additives such as soluble carbohydrate is highly recommended in fermentation (McDonald et al. 1991).

CONCLUSION

Fermentation was the most effective technique to reduce tannin concentration yet improves nutrient value of *C. odorata*. Fermentation decreased total tannin concentration by 62%, reduced crude fiber from 127 g/kg DM into 86 g/kg DM (32% improvement), and increased crude protein from content from 175 g/kg DM to 290 g/kg DM (60% improvement). This study showed that anaerobic fermentation is the best method

to treat *C. odorata* to improve its biological value as a potential animal feed.

REFERENCES

Atanassova M, Christova-Bagdassarian V. 2009. Short Communication: Determination of tannins content by titrimetric method for comparison of different plant species. J Univ Chem Technol Metallurgy. 44:413-415.

Akinmoladun AC, Obuotor EM, Farombi EO. 2010. Evaluation of antioxidant and free radical scavenging capacities of some Nigerian indigenous medicinal plants. J Med Food. 13:444-451.

Bhatta R, Uyeno Y, Tajima K, Takenaka A, Yabumoto Y, Nonaka I, Enishi O, Kurihara M. 2009. Difference in the nature of tannins on *in vitro* ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J Dairy Sci. 92:5512-5522.

Bira GA, Mullik ML, Jelantik IGN, Maranatha G, Mullik YM, Sudarma I-MA, Dahlanuddin. 2015. Incremental levels of *C. odorata* in complete diet does not impar intake, rumen fermentation and microbial crude protein synthesis in cattle. Proceeding 3rd International Seminar on Animal Industry. Bogor (Indones): Bogor Agricultural University. p. 229-232.

Borchani C, Besbes S, Masmoudi M, Blecker C, Paquot M, Attia H. 2011. Effect of drying methods on physico-chemical and antioxidant properties of date fibre concentrates. Food Chemist. 125:1194-1201.

Djordjevic S, Pace CP, Stankovich MT, Kim JJ. 1995. Three-dimensional structure of butyryl-CoA dehydrogenase from Megasphaera elsdenii. Biochemistry. 34:2163-2171.

Gemede BS, Hassen A. 2015. Effects of tannin and species variation on *in vitro* digestibility, gas and methane production of tropical browse plants. AJAS. 28:188-199.

Hagerman AE. 1992. Tannin-protein interaction. In: Chi-Tang H, ChangYL, Mou-Tuan H, editors. Phenolic compounds in food and their effect on health I. Washington DC (USA): American Chemical Society. p. 237-247.

Hagerman AE. 2002. Tannins chemistry. Tannin Handbook. Oxford (USA): Miami University.

Hai PV, Everts H, Tien DV, Schonewille JT, Hendriks WH. 2012. Feeding *Chromonaela odorata* during pregnancy to goat dams affects acceptance of this feedstuff by their offspring. Appl Anim Behav Sci. 137:30-35.

Haslam E. 1989. Chemistry and significance of condensed tannins. In: Plant polyphenols. Hemingway RW, Karchesy JJ, editors. Plenum. Cambridge (UK): Cambridge University Press.

Hue KT, Van DTT, Ledin I, Spörndly E, Wredle E. 2010. Effect of feeding fresh, wilted and sun-dried foliage from cassava (*Manihot esculenta* Crantz) on the performance of lambs and their intake of hydrogen cyanide. Livest Sci. 131:155-161.

Kana-Hau D, Nenobais M, Nulik J, Katipana NGF. 2005. Pengaruh probiotik terhadap kemampuan cerna mikroba rumen sapi Bali. Mathius I-W, Bahri S, Tarmudji, Prasetyo LH, Triwulanningsih E, Tiesnamurti B, Sendow I, Suhardono. Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner. Bogor (Indones): Pusat Penelitian dan Pengembangan Peternakan. p. 171-180.

Khanbabaee K, Van Ree T. 2001. Tannins: Classification and definition. Nat Prod Rep. 18:641-649.

Makkar HPS, Becker K. 1996. Effect of pH, temperature, and time on inactivation of tannins and possible implications in detannification studies. J Agric Food Chem.44:1291-1295.

Makkar HPS. 2003. Quantification of tannins in three and shrub foliage. A laboratory manual. Dordrech (Nethrelands): Kluwer Academic Publisher.

McDonald P, Henderson AR, Heron SJE. 1991. The biochemistry of silage. 2nd ed. Marlow (USA): Chalcombe Publication.

McSweeney CS, Palmer B, Mcneil DM, Krause DO. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. Anim Feed Sci Technol. 91:83-93.

Mullik ML. 2002. Laporan penelitian: Strategi pemanfaatan semak bunga putih (*Chromolaena odorata*) untuk meningkatkan produksi ternak dan pendapatan peternak di daerah lahan kering. Kerjasama Fakultas Peternakan Universitas Nusa Cendana dan Kementrian Riset dan Teknologi Republik Indonesia melalui Riset Pengembangan Kapasitas.

Mullik ML, Jelantik I-G, Mullik YM, Dahlanuddin, Wirawan I-GO, Permana B. 2014. Pemanfaatan semak bunga putih (*Chromolaena odorata*) sebagai pakan lokal sumber protein untuk ternak sapi: konsumsi, daya cerna dan fermentasi rumen. Zain M, Sowmen S, Rusfidra, Rusdimansyah, Wati R, Amizar R, editors. Prosiding Seminar Nasional III HITPI. Padang (Indones): Universitas Andalas. p. 312-320.

Ngozi II, Ikewuchi CJ, Ikewuchi. 2009. Chemical profile of *Chromolaena odorata* Linn (King and Robinson) leaves. Pak J Nutr. 8:521-524.

Onkaramurthy M, Veerapur VP, Thippeswamy BS, Madhusudana Reddy TN, Rayappa H, Badami S. 2013. Anti-diabetic and anti-cataract effects of *Chromolaena odorata* Linn in streptozotocin-induced diabetic rats. J Ethnopharmacol. 145:363-372.

Osawa R, Walsh TP. 1993. Effects of acidic and alkaline treatments on tannic acid and its binding property to protein. J Agric Food Chem. 41:704-707.

Patra AK, Saxena J. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J Sci Food Agric. 91:24-37.

Rakić S, Maletić R, Perunović M, Svrzić G. 2004. Influence of thermal treatment on tannin content and antioxidation effect of oak acorn quercus cerris extract. J Agric Sci. 49:97-107.

Roger T, Léopold TN, Funtong MC. 2015. Nutritional properties and antinutritional factors of corn paste (*kutukutu*) fermented by different strains of lactic acid bacteria. Int J Food Sci. 2015:1-13.

Taylor JR, Duodu KG. 2014. Effects of processing sorghum and millets on their phenolic phytochemicals and the implications of this to the health-enhancing properties of sorghum and millet food and beverage products. 95:225-237.

Wina E. 2010. Utilization of tannin containing shrub legumes for small ruminant production in Indonesia. Wartazoa. 20:21-30.

Wina E, Tangedjaja B, Palmer B. 2000. The effect of drying condition on condensed tannin estimater (CT) in *Calliandra calothyrsus.* Brooker JD, editor. Tannins in Livestock and Human Nutrition. ACIAR Proceeding. 92:106-110.

Follicular Dynamic and Repeatability of Follicular Wave Development in Peranakan Ongole (PO) Cattle

Imron M[1], Supriatna I[2], Amrozi[2], Setiadi MA[2]

[1]*Livestock Embryo Center, Cipelang, Bogor PO Box 485 Bogor 16004*
[2]*Division of Reproduction and Toxicology, Department of Pathology and Reproduction Clinic, Faculty of Veterinary, Bogor Agricultural University, Jl. Agatis, Kampus IPB Dramaga-Bogor 16680*
E-mail: hibbanie@gmail.com

(received 11-01-2016; revised 29-02-2016; accepted 07-03-2016)

ABSTRAK

Imron M, Supriatna I, Amrozi, Setiadi MA. 2016. Dinamika folikel dan repeatabilitas pertumbuhan gelombang folikel pada sapi peranakan ongole (PO). JITV 21(1): 26-33. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1349

Perlakuan superovulasi pada sapi PO (*Bos indicus*) memiliki respon yang rendah dibandingkan dengan rumpun *Bos taurus* yang mungkin berhubungan dengan perbedaan dinamika folikel antara kedua rumpun sapi. Penelitian ini dilakukan untuk mengetahui dinamika folikel dan repeatabilitas pola pertumbuhan gelombang folikel pada sapi PO. Pada tahap awal, penelitian menggunakan 9 ekor sapi PO berumur 5-7 tahun. Pengamatan dilakukan dengan menggunakan ultrasonografi (USG) setiap hari. Hasil pengamatan menunjukkan sapi PO memiliki pola 3 (66%) dan 4 (34%) gelombang pertumbuhan folikel dalam satu siklus. Gelombang folikel pertama dari pola gelombang 3 dan 4 terlihat berturut-turut hari ke 0,4±0,9 dan 1,4±1,1 relatif terhadap terjadinya ovulasi. Gelombang kedua terjadi berturut-turut hari ke 9,8±1,5 dan 7,4±1,9 pada pola 3 dan 4 gelombang, relatif terhadap terjadinya ovulasi. Pola 3 gelombang memiliki durasi folikel dominan lebih panjang (11,6±1,5 hari) dibandingkan pola 4 gelombang (10±2,9 hari). Kecepatan pertumbuhan folikel dominan tidak berbeda antara pola 3 dan 4 gelombang folikel (0,87±0,23 dan 0,94±0.25 mm/hari). Diameter folikel ovulatori antara pola 3 dan 4 gelombang folikel tidak berbeda yaitu berturut-turut 12,24±0,71 dan 12,30±0,22 mm. Diameter CL juga tidak berbeda antara pola 3 dan 4 gelombang folikel, yaitu 18,94±0,47 dan 19,44±0,87 mm. Pengamatan repeatabilitas pola gelombang yang dilakukan menggunakan 6 ekor menunjukkan sapi PO memiliki repeatabilitas tinggi pada pola gelombang (0,88) dan jumlah folikel yang berkembang (0,91). Penelitian ini menunjukkan data dinamika perkembangan folikel, pola gelombang dan repeatabilitasnya yang diharapkan membantu mendesain protokol perlakuan superovulasi atau teknologi reproduksi yang lain berbasis dinamika folikel sapi PO agar memperoleh respon yang lebih baik.

Kata Kunci: Sapi PO, Gelombang Folikel, Repeatabilitas, Interovulatori Interval

ABSTRACT

Imron M, Supriatna I, Amrozi, Setiadi MA. 2016. Follicular dynamic and repeatability of follicular wave development in Peranakan Ongole (PO) Cattle. JITV 21(1): 26-33. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1349

Superovulation treatment on PO cattle (Bos indicus) was less responsive compared to *Bos taurus* breed. It might due to the difference of their follicular dynamic. This study was conducted to investigate the follicular dynamics and its repeatability in PO cattle. Follicular dynamics observations conducted on 9 cows trough ultrasound scanning every day. Observations of wave patterns repeatability were performed in 6 cows which its wave pattern already known on the next consecutive IOI. Research result indicated that PO cattle had 3 (66%) and 4-waves (34%) pattern. The first wave of 3 and 4-waves pattern emerged on day -0.4+0.9 and 1.4+1.1 respectively. The second wave of 3 and 4-wave pattern emerged on day 9.8+1.5 and 7.4+1.9 respectively. The pattern of 3 waves has a longer follicle dominant duration (11.6+1.5 day) in the first wave of estrous cycle, compared with 4 waves pattern (10+2.92 and 7+1.00 day respectively). The growth rate of dominant follicle was not different significantly between the 3 and 4-waves pattern (0.87+0.23 and 0.94+0.25 mm/day respectively). Similarly, ovulatory follicle diameter between 3 and 4-waves pattern was also not different significantly (12.24+12.34 and 12.30+12.23 mm respectively). Observation of wave patterns repeatability in 6 PO cows indicated that PO cattle had high repeatability in follicular wave pattern (0.88) and the number of growing follicle was 0.91. This study resulted data for dynamic of follicular development, wave pattern, its repeatability which be expected to design the protocol of superovulation treatment or other reproduction technologies based on follicular dynamic to improve its result in PO cattle.

Key Words: PO Cattle, Follicular Wave, Repeatability, Interovulatory Interval

INTRODUCTION

Follicular wave was identified by growth of a small group follicle with diameter by 3-4 mm (Taylor & Rajamahendran 1991). Wave growth pattern of follicle might be observed in prepubertal (Melvin et al. 1999), during pregnancy (lactating period) (Taylor & Rajamahendran 1991), post-partum (Murphy et al. 1990), and during estrus cycle (Roche et al. 1999). Growth of follicular wave was began with increase of concentration of FSH serum and followed by sudden growth of 8-41 small follicles in 2-3 days (Ginther et al. 1997). One follicle would be selected to continuously grow to be dominant follicle (DF), meanwhile the smaller follicles would be subordinate follicle (SF) and would undergo an atresia. If there was a luteolysis in the growing-phase DF, follicle would undergo final maturation process continued by ovulation. If there was no lutheolysis in the growing DF, the DF would undergo an atresia (Vasenna et al. 2003; Jaiswal et al. 2004; Adam et al. 2008).

Most of cattle breed showed 2 and 3 follicular wave pattern in one estrous cycle (Adam et al. 2008) although sometimes showed 1, 4, and 5 follicular wave pattern (Bleach et al. 2004; Viana et al. 2000). Follicular dynamic was one of research subjects which have been much studied in Europe breed (*Bos taurus*) but information of zebu cattle (*Bos indicus*) was very limited. Bó et al. (2003) has reviewed that reproduction characteristic of *B. indicus* cattle was different from *B. taurus* such as in length of estrous cycle, estrous time, estrous behavior, growth of dominant follicle and CL.

Superovulation program at Cipelang Livestock Embryo Center (LET) performed to PO cattle (*Bos indicus*) as donor showed lower ration of transferable embryo per total embryo collected (1.5 embryos) than Angus, Simmental and Limousine cattle (*Bos taurus*) which had ratio by 4.75, 3.37 and 2.96 embryos respectively (BET 2012). Hormone injection in superovulation was undifferentiated between donor from *Bos taurus* (Simmental and Limousine cattle) and donor from *Bos indicus* (PO cattle). Bó & Mapletoft (2014) said that response of superovulation would be optimal if follicle super-stimulation treatment was started in the beginning of follicular growth wave emergence. One day earlier or later gonadotropin treatment from initial follicular wave would decrease superovulation response compared to starting treatment right at the time of the initial follicular wave emergence (Bó et al. 2008). It was assumed that there was a relation of difference of reproduction characteristic between both cattle breeds especially in follicular wave pattern with the obstacles faced in superovulation program in PO cattle. Information of characteristic of ovulary dynamic and its repeatability are needed to optimize superovulation program in PO cattle.

This study was conducted to determine characteristic of ovulary dynamic during estrous cycle and repeatability rate of follicular growth wave pattern in Peranakan Ongole (PO) cattle.

MATERIALS AND METHODS

Materials

In this study 9 five-seven years old PO cows with body weight of 375-450 kg were used. All cows had Body Condition Value (BCV) ranged in 2.7-3.2 of scale 1-5 with normal estrous cycle. Diet was provided as 30-40 kg of grass per head per day and 2-3 kg of commercial diet per head per day.

Ovulation synchronization

This study was started by ovulation synchronization to synchronize initial observation of ovulary dynamic through installation of intra-vaginal progesterone preparate (Cuemate®, consisted of 1.56 mg progesterone in 2 pod silicon, Bioniche Animal Health (A/Asia) Pty.Ltd., Australia) followed by injection of 100 µg GnRH (Fertagyl®, Intervet Schering-Plough Animal Health, German) in the first day. Cuemate® was entered into vagina for 7 days using special applicator oiled by isotonic gel. PGF2α (Prostavet® C, 5 mg of etiprostone per 2 ml of solution; Virbac Animal Health, France) was injected intramuscularly along with Cuemate® releasing in vagina. Two days later, 100 µg GnRH (Fertagyl®, Intervet Schering-Plough Animal Health, German) was added intramuscularly to induce ovulation synchronization.

Observation of follicular dynamic

Observation of follicular dynamic was performed once a day at the same time and operator using portable ultrasonic (EasyScan Lite, England) equipped with goggle monitor and probe with dynamic frequency by 4-8 Mhz. The main unit of ultrasound was connected to computer and data were recorded in video version. Measurement of diameter of follicle was conducted using Microsoft image tool. Daily observation using ultrasound was started when PO cattle injected by PGF2α. One inter-ovulatory interval (IOI) was defined as time period between 1 ovulation with the next ovulation. First ovulation observed after ovulation synchronization was assumed as day-0 in 1 IOI.

In the initial step, observation was conducted to obtain characteristic of follicular dynamic and for wave pattern in 1 IOI mapping. Nine cows were used in the initial study. To obtain repeatability of follicular wave growth, observation of follicular dynamic was continued in the next1 IOI without a break using 6 cows

(3 cows with 3-wave pattern and 3 cows with 4-wave pattern).

Data analysis

One follicular wave was defined as duration of follicular growth started from 4 mm diameter until the dominant follicle back to its original size. Follicle reaching the biggest size from the same wave was stated as dominant follicle (DF), meanwhile smaller follicle was defined as subordinate follicle (SF). Follicular growth phase was a period of follicle detected in 4 mm of diameter until the time the follicle did not grow anymore. Regression phase was started at the time follicle diameter decreased until getting hard to be detected individually. Static phase was defined as a period between the last days of growth phase and first day of regression phase. Data were divided according to pattern of follicular wave amount observed in 1 IOI (3 and 4-wave pattern group) and was tested by Independent-Sample T Test. Repeatability (0-1 value) was defined as proportion of total variance which was able to contribute to individual variance, and was calculated by formula: individual σ^2/(individual σ^2 + σ^2 error) (Burns *et al*. 2005). Component of variance for repeatability was estimated using ANOVA with single factor. Percentage of follicular ovulation was amount of ovulation in the left or right ovary divided by total follicular ovulation observed. Percentage of follicular wave occurrence in ovary was amount of follicular wave occurred in the right or left ovary divided by total follicular wave observed.

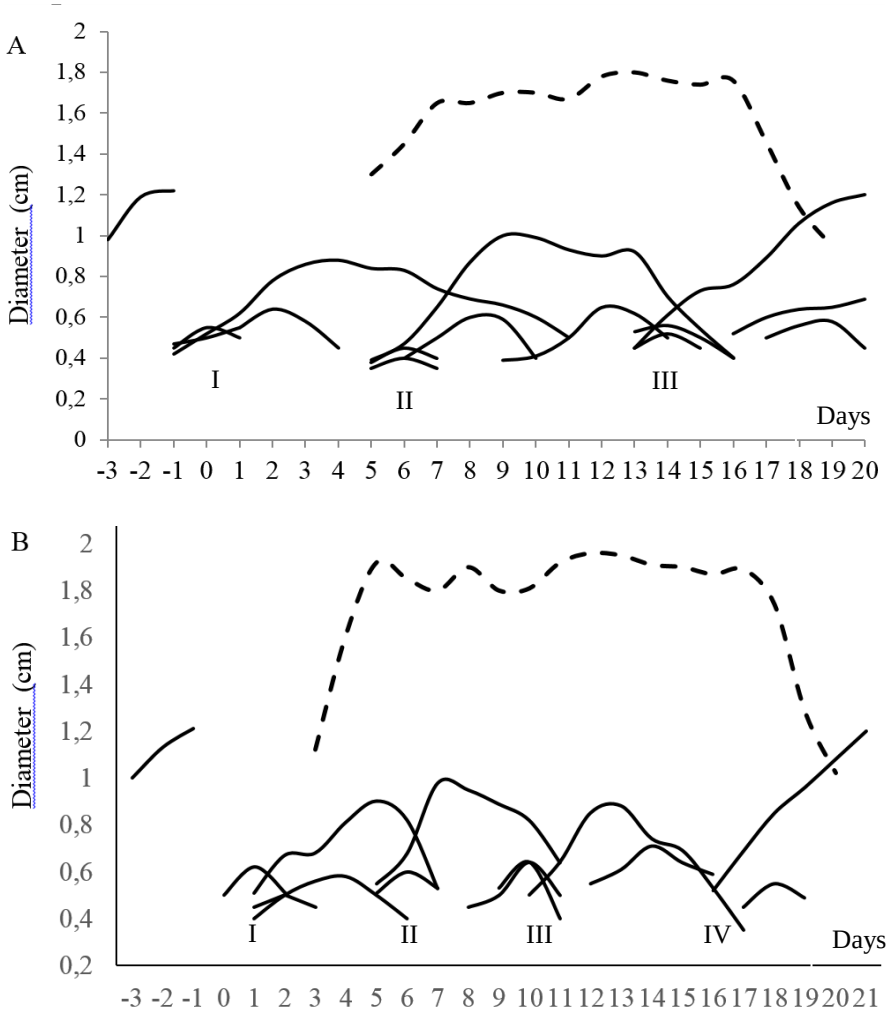

Figure 1. Growth pattern of follicular wave and CL in PO cattle with 3-follicular wave (A) and 4-follicular wave (B). Description: dotted line is CL growth pattern, solid line is follicular growth pattern.

RESULT AND DISCUSSION

Based on daily observation of follicular dynamic, 6 cows (66%) showed 3-follicular wave pattern and the 3 (33%) showed 4-follicular wave pattern (Figure 1) and there was no 2-wave pattern. Melia et al. (2014) reported that 6 PO cattle showed 3-wave pattern. In other study, Viana et al. (2000) reported that there was only 1 (6%) of 15 Gyr cattle (*B. indicus*) which had 2-follicular wave pattern, the rest 60% and 26.7% had 3 and 4-follicular wave pattern, respectively.

Wave amount in 1 cycle was not affected by cattle breed (Adam et al. 2008), however Bó et al. (2003) reported that there was 3-wave pattern in poor nutrition and heat-stressed cattle. There was different in wave pattern proportion of heifer and cow in dairy cattle (Wolfenson et al. 2004). In other study, Adam et al. (2008) said that the most heifers (65%) of Nellor cattle (*B. indicus*) showed 3-wave, while most of cows (83%) showed 2-wave pattern. Satheshkumar et al. (2015) reported that 3-wave pattern emerged more frequently in the winter than the 2-wave pattern, and conversely in the summer.Follicular wave growth was initiated in day -0.4±0.9 and 1.4±1.1 relative against ovulation in cattle with 3 and 4 follicular wave. The next follicular wave started to grow in day 8.1±1.5 and 7.4±1.9 for the 3 and 4-wave pattern (Table 1). Three-wave pattern had the longest dominant duration (11.6+1.5 days) on the first wave and shorter on 4-wave pattern (10.0±2.9). This was in line with Adam et al. (2008) who said that there was a highly correlation between dominant duration with its wave amount pattern. Shorter dominant duration would increase the number of wave in 1 IOI. In other study, Jaiswal et al. (2009) said that dominant duration on the first wave in IOI might be used to predict wave amount pattern. Diameter of dominant follicle (DF) of each follicular wave in 3 and 4-wave pattern was not significantly different even diameter of dominant follicle tended to wane by increase of wave amount in 1 IOI (Table 1). Viana et al. (2000) said that decrease of diameter of dominant follicle and its dominant duration was associated with increase of wave amount in 1 estrous cycle. Bó et al. (2003) reported that the number of *B. indicus* (16%) which had 4 follicular wave pattern was higher than *B. taurus* (0%) and was allegedly due to a consequence of smaller diameter of dominant follicle and shorter dominant duration of *B. indicus*. High concentration estradiol suppressed LH impulse inducing synthesis of progesterone of luteal cells (Goodman et al. 1981; William et al. 1978), so that it was expected might affect function of luteal and its lifetime (Jaiswal et al. 2009).

However, on the third wave of 3-wave pattern which was an ovulatory follicle had significantly wider diameter than the third wave of 4-wave pattern (Table 1). As well it had wider diameter of ovulatory follicle than that non-ovulatory follicle in the same wave pattern. Progesterone produced by CL would suppress concentration of estradiol and growth rate of dominant follicle (Ramana et al. 2013; Junior et al. 2010), so that follicle growing during luteal phase had smaller diameter than ovulatory follicle growing during luteolysis.

One IOI cycle in this study tended to longer by increase of 3 and 4 wave pattern by 22±1.6 and 22.8±2.9 respectively (Table 2). It possibly related to size of follicular diameter in each wave pattern. Goff et al. (2004) said that estradiol in the first wave of IOI regulated time of oxytocin receptor emergence in endometrium that eventually managing prostaglandin production time to stimulate the luteolisis. There was a suspicion that size of follicular diameter on the first

Table 1. Characteristic per dominant follicular wave of PO cattle with 3 and 4 follicular wave in 1 IOI

Observation	Follicular wave			
	I	II	III	IV
Onset follicle (day-):				
3-wave	-0.4±0.9	8.1±1.5	15±2.1	-
4-wave	1.4±1.1	7.4±1.9	12.2±1.5	16.2±3.2
Dominant duration (day)				
3-wave	11.6±1.5	11±2.3	6.8±1.3	-
4-wave	10±2.9	8.8±1.6	7.7±1.5	6.6±0.9
Diameter of DF (mm)				
3-wave	10.05±0.43	9.26±0.94	12.24±0.71^{a*}	-
4-wave	9.42±0.44	8.84±1.15	9.1±1.44^{b}	12.30±0.22

Different *superscript* in the same column and characteristic shows significant different in P<0.05. *: Diameter of ovulatory follicle

Table 2. Characteristic of dynamic of dominant follicle, subordinate follicle and corpus lutheum (CL) of PO cattle with 3 and 4 follicular waves

Characteristic	3-waves	4 -waves
Dominant follicle		
The number of IOI	7	5
IOI length (day)	22.0±1.6	22.8±2.9
Growth rate (mm/day)	0.87±0.23	0.94±0.25
Static phase (day)	3.7±2.0	2.7±1.9
Atresia rate (mm/day)	0.83±0.21	O.91±0.31
Follicle growth to ovulation (day)	7.6±0.6	7.4±1.1
Diameter of ovulatory follicle (mm)	12.24±0.71	12.30±0.22
Diameter when deviation (mm)	6.25+0.37	6.44+0.44
Growth rate of subordinate follicle velocity (mm/day)	0.83±0.25	0.72±0.33
Diameter of CL on day-10 (mm)	18.94+0.47	19.44+0.87

wave of IOI affecting pattern of follicular wave amount formed (Adam et al. 2008; Jaiswal et al. 2009). However, Boer et al. (2011) said that mechanism of wave pattern forming was still unclear and was allegedly involving more complex follicular growth regulation.

In this study, growth rate of dominant follicle was not significantly different between 3 and 4-wave (0.87+0.23 and 0.94+0.25 mm/day, respectively) (Table 2). As well DF growth rate was not significantly different from SF until the follicular deviation. This was in line with Adam et al. (2008) who said that a group of follicle had the same growth rate at the beginning of growth until one follicle was selected to continuously grow to become a DF.

Initial of growth difference between the two biggest follicles was defined as follicular deviation (Ginther et al. 2003). In this study, deviation was occurred after the selected follicle reached diameter of 6.25+0.37 and 6.44+0.44 in 3 and 4-wave respectively (Table 2). That was lower compared to follicular deviation in *B. taurus* breed by 8.5-9.0 mm (Ginther & Hoffman et al. 2014; Sartori et al. 2001). This result was in line with Sartorelli et al. (2005) reporting that Nellor cattle (*B. indicus*) had smaller follicular diameter during deviation and ovulation than *B. taurus*. *B. indicus* breed had smaller follicle dominant than *B. taurus*, so that follicle dominant in *B. indicus* became smaller during follicular deviation (Bó et al. 2003).

When the DF depended on LH, there were only 2 possibilities: ovulation or regression (Lucy 2007). DF unexposed by LH surge would remind at a certain period (static phase) and then regressed (Valdez et al. 2005). During this period, DF would depend on growth factor supporting transition of G1 into S phase of cellular cycle and prevented apoptosis in granulose cells (Quirk et al. 2004). At the certain time in static phase, DF would lose its dominant function even morphologically this follicle was the biggest size (Ireland et al. 2000). In this study, cows with 3-wave pattern had longer static phase by 3.7+1.9 days than 4-wave pattern by 1.9+1.4 days.

Ovulatory follicle of 3 and 4-wave had no significant difference diameter by 12.24+0.34 and 12.30+0.23 mm, respectively (Table 2). Growth length follicle into ovulation was also not different between 3 and 4-wave pattern (18.94+0.47 and 19.44+0.87 mm, respectively) (Table 2). As well CL diameter in day-10 after ovulation was not significantly different between 3 and 4-wave pattern (18.94+0.47 and 19.44+0.87 mm, respectively).

Wave amount pattern in this study had high repeatability value by 0.88 in the same individual (Table 3). It was in line with Jaiswal et al. (2009) who said that wave pattern had high repeatability value in crossed Hereford (*B. taurus*) cattle. Sichtar et al. (2010) reported almost equal proportion between individual experiencing a change or not in wave amount pattern in the same individual of dairy cattle.

The number of follicle growing in this study had high repeatability value by 0.91 in the same individual. Another studies also reported that the number of follicle recruited into one wave had high repeatability value in the same individual, but was varied in the different individual (Santos et al. 2014; Ireland et al. 2007; David et al. 2005). Singh et al. (2004) reported that the high

Table 3. Value of characteristics of follicular dynamic observed in PO cattle

Observation	Value
Repeatability of (3 and 4) follicular wave pattern respectively in 2 IOI in the same individual	0.88
Repeatability of follicle amount growing in the same individual	0.91
Average follicle amount at the beginning of follicular wave growth	27.3±9.4
Percentage of ovulation in the right and left ovary	72% and 28%
Percentage of follicular wave in the right and left ovary	52% and 48%
Repeatability of follicular wave in the right and left ovary	0.14 and 0.10

number of antral follicle had a positive correlation to gonadotropin treatment during superovulation and produced more oocyte and transfer-feasible embryo. So that, characteristic of repeatability of high number of PO cattle might be used as parameter of donor selection to increase effectiveness of superovulation. In this study, ovulation in right ovary was higher (72%) than in the left (Table 3). It was in line with Vasenna et al. (2003) and Nation et al. (1999) who reported that ovulation more often occurred in the right ovary than in the left. However, Purwantara et al. (2006) and Ginther et al. (1989) reported balance ovulation in the right and left ovary. In other hand, percentage of the number of follicular wave formed between the right and left ovary was practically balance (52% and 48% respectively) showing that actually, right and left ovary had the same activity in folliculogenesis.

Repeatability of follicular wave emergence in the same ovary was too low both in the right and left ovary (0.14 and 0.10, respectively) showing a process of randomly follicular wave forming in the right and left ovary. This was different from Vasenna et al. (2003) reporting that activity of the right ovary was higher than the left. It was just 1 from a pair of follicle would be selected to be DF both in ipsilateral or contralateral position against the biggest subordinate follicle.

This study provided basic data of follicular dynamic and its repeatability in PO cattle based on USG observation. A better understanding of follicular dynamic was very useful in its utilization for purposes with more specific reproduction management such as in superovulation program. Some field studies had been conducted to implement superovulation program to other native cattle (Bali, Madura, and Aceh cattle) with standard superovulation protocol as applied in *Bos taurus* breed. However, until now response of superovulation produced was not as expectation. This indicated the urgent of understanding of follicular dynamic characteristics of each native cattle breed to be used as a basic data in implementation reference of reproduction technology, especially that superovulation program.

CONCLUSION

This study showed that characteristic of follicular dynamic of PO cattle was dominated by 3-waves pattern in 1 estrous cycle. Repeatability of wave pattern and follicle number had high value in the same individual. Those characteristics might be used for reproduction management of donor cattle to increase superovulation effectiveness based on individual information.

REFERENCES

Adams GP. 1999. Comparative patterns of follicle development and selection in ruminants. J Reprod Fertil. 54:17-32.

Adams GP, Jaiswal R, Singh J Malhi P. 2008. Progress in understanding ovarian follicular dynamics in cattle. Theriogenology. 69:72-80.

[BET] Balai Embrio Ternak. 2012. Laporan tahunan: Evaluasi produksi dan transfer embrio. Bogor (Indones): Balai Embrio Ternak. hlm. 31-36.

Bleach ECL, Glencross RG, Knight PG. 2004. Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles. Reproduction. 127:621-629.

Boer HMT, Röblitz L, Stötzel C, Veerkamp RV, Kemp B, Woelders H. 2011. Mechanisms regulating follicular wave patterns in the bovine estrous cycle investigated with a mathematical model. J Dairy Sci. 94:5987-6000.

Bó GA, Baruselli PS, Mart MF. 2003. Pattern and manipulation of follicular development in *Bos indicus* cattle. Anim Reprod Sci. 78:307-326.

Beg MA, Bergfelt DR, Kot K, Ginther OJ. 2002. Follicle selection in cattle: dynamics of follicular fluid factors during development of follicle dominance. Biol Reprod. 66:120-126.

Burns DS, Jimenez-Krassel FJ, Ireland JJ. 2005. Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association

with serum follicle-stimulating hormone concentrations. Biol Reprod. 73:54-62.

David SB, Jimenez-Krassel F, Ireland JLH, Knight PG, James J. 2005. Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. Biol Reprod. 73:54-62.

Ereno RL, Pupulim AG, Loureiro B, Favoreto MG, Castilho ACS, Buratini J, Barros CM. 2013. The effect of nutrition onovarian follicle population and plasma anti-Mullerian hormone con-centration in Aberdeen Angus heifers. Reprod Fertil Dev. 25:236.

Fair T, Hulshof SCJ, Hyttel P, Greve T, Boland M. 1997. Oocyte ultrastructure in bovine primordial to early tertiary follicles. Anat Embryo. 195:327-336.

Fortune JE, Cushman RA, Wahl CM, Kito S. 2000. The primordial to primary follicle transition. Mol Cell Endocrinol. 163:53-60.

Ginther OJ, Knopf L, Kastelic JP. 1989. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J Reprod Fertil. 87:223-230.

Ginther OJ, Kot K, Kulick LJ, Wiltbank MC. 1997. Emergence and deviation of follicles during the development of follicular waves in cattle. Theriogenology. 48:75-87.

Ginther OJ, Hoffman MM. 2014. Intraovarian effect of dominant follicle and corpus luteum on number of follicles during a follicular wave in heifers. Theriogenology. 82:169-175.

Ginther OJ, Beg MA, Donadeu FX, Bergfelt DR. 2003. Mechanism of follicle deviation in monovular farm species. Anim Reprod Sci. 78:239-257.

Goodman RL, Bittman EL, Foster DL, Karsch FJ. 1981. The endocrine basis for the synergistic suppression of luteinizing hormone by estradiol and progesterone. Endocrinology. 109:1414-1417.

Goff AK. 2004. Steroid hormone regulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle. Biol Reprod. 71:11-16.

Ireland JJ, Mihm M, Austin E, Diskin MG, Roche JF. 2000. Historical perspective of turnover of dominant follicles during the bovine estrous cycle: key concepts, studies, advancements, and terms. J Dairy Sci. 83:1648-1658.

Ireland JJ, Ward F, Jimenez-Krassel F, Ireland JLH, Smith GW, Lonergan P, Evans ACO. 2007. Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. Human Reproduction. 22:1687-1695.

Jaiswal RS, Singh J, Marshal L, Adam GP. 2009. Repeatability of 2-wave and 3-wave patterns of ovarian folicular development during the bovine estous cycle. Theriogenology. 71:81-90.

Junior CI, Sa-Filho OG, Perez RFG, Aono FHS, Day ML, Vasconcelos JLM. 2010. Reproductive Performance of prepubertal Bos indicus herifer after progesteron-based treatments. Theriogenology. 74:903-911.

Kanitz W. 2003. Follicular dynamic and ovulation in cattle: A review. Arch Tierz. 46:187-198.

Melia J, Amrozi, Tumbelaka LI. 2014. Dinamika ovarium sapi endometritis yang diterapi dengan gentamicine, flumequine dan analog prostaglandin F2 alpha ($PGF_2\alpha$) secara intra uterus. J Kedokteran Hewan. 8:111-115.

Melvin EJ, Lindsey BR, Quintal-franco J, Zanella, Fike KE, Van Tassell CP, Kinder JE. 1999. Circulating concentrations of estradiol, luteinizing hormone, and follicle-stimulating hormone during waves of ovarian follicular development in prepubertal cattle. Biol Reprod. 60:405-412.

Mihm M, Baker PJ, Ireland JLH, Smith GW, Coussens PM, Evans ACO, Ireland JJ. 2006. Molecular evidence that growth of dominant follicles involves a reduction in follicle stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. Biol Reprod. 74:1051-1059.

Monniaux D, Barbey S, Rico C, Fabre S, Gallard Y, Larroque H. 2010. Anti-Müllerian hormone: a predictive marker of embryo productionin cattle?. Reprod Fertil Dev. 22:1083-1091.

Mossa F, Walsh SW, Butler ST, Berry DP, Carter F, Lonergan P, Smith GW, Ireland JJ, Evans ACO. 2012. Low numbers of ovar-ian follicles ≥3 mm in diameter are associated with low fertility indairy cows. J Dairy Sci. 95:2355-2361.

Murphy MG, Boland MP, Roche JF. 1990. Pattern of follicular growth and resumption of ovarian activity in post-partum beef suckler cows. J Reprod Fertil. 90:523-533.

Nation DP, Burke CR, Rhodes FM, Macmillan KL. 1999. The inter-ovarian distribution of dominant follicles is influenced by the location of the corpus luteum of pregnancy. Anim Reprod Sci. 56:169-176.

Lucy MC. 2007. The bovine dominant ovarian follicle. J Anim Sci. 85:89-99.

Lussier JG, Matton P, Dufour JJ. 1987. Growth rates of follicles in the ovary of the cow. J Reprod Fertil. 81:301-307.

Parrott JA, Skinner MK. 2000. Kit ligand actions on ovarian stromal cells: Effects on theca cell recruitment and steroid production. Mol Reprod Dev. 55:55-64.

Purwantara B, Hoier R, Schmidt M, Greve T. 2006. Preovulatori changes and ovulation in cattle undergoing spontaneous or clopprostenol-induced luteolyis. Biotropia. 13:75-84.

Quirk SM, Cowan RG, Harman RM, Hu CL, Porter DA. 2004. Ovarian follicular growth and atresia: The

relationship between cell proliferation and survival. J Anim Sci. 82:40-52.

Ramana KV, Rao KV, Supriya K, Rajanna N. 2013. Postpartum ovarian follicular dynamics and estrus activity in lactating Ongole cows. J Res Angrau. 41:51-55.

Roche JF, Austin EJ. Ryan M, O'rourke M, Mihm M, Diskin MG. 1999. Regulation of follicular waves to maximize fertility in cattle. J Reprod Fertil. 54:61-71.

Santos KC, Santos GMG, Siloto LS, Morotti F, Marcantonio TN, Seneda MM, 2014. Comparison of antral and preantral ovar-ian follicle populations between *Bos indicus* and *Bos taurus* cows with high or low antral follicles counts. Reprod Dom Anim. 49:48-51.

Sartori R, Fricke PM, Ferreira JCP, Ginther OJ, Wiltbank MC. 2001. Follicular deviation and acquisition of ovulatory capacity in bovine follicles. Biol Reprod. 65:1403-1409.

Sartorelli ES, Carvalho LM, Bergfelt DR, Ginther OJ, Barros CM. 2005. Morphological characterization of follicle deviation in Nelore (*Bos indicus*) heifers and cows. Theriogenology. 63:2382-2394.

Satheshkumar S, Brindha K, Roya A, Devanathan TG, Kathiresan D, Kumanan K. 2015. Natural influence of season on follicular, luteal, and endocrinological turnover in Indian crossbred cows. Theriogenology. 84:19-23.

Sichtar J, Tolman R, Rajmon R, Klabanová P, Berka P, Volek J. 2010. A comparison of the follicular dynamics in heifers of the Czech Fleckvieh and Holstein breeds. Czech J Anim Sci. 55:234-242.

Silva-Santos KC, Santos GMG, Siloto LS, Morotti F, Marcantonio TN, Seneda MM. 2014. Comparison of antral and preantral ovarian follicle populations between *Bos indicus* and *Bos indicus-taurus* cows with high or low antral follicles counts. Reprod Dom Anim. 49:48-51.

Siddiqui MAR, Ginther OJ. 2014. Switching of largest follicle from dominant to subordinate status when follicle and CL are in same ovary in heifers. Theriogenology. 82:259-265.

Taylor C, Rajamahendran R. 1991. Follicular dynamics, corpus luteum growth and regression in lactating dairy cattle. Canadian J Anim Sci. 71:61-68.

Valdez KE, Cuneo SP, Turzillo AM. 2005. Regulation of apoptosis in the atresia of dominant bovine follicles of the first follicular wave following ovulation. Reproduction. 130:71-81.

Viana JHM, Ademir MF, Wanderlei FS, Almeida LS, Camargo. 2000. Follicular dynamics in zebu cattle. Pesq Agropec Bras. 35:2501-2509.

Williams MT, Marsh JM. 1978. Estradiol inhibition of luteinizing hormone-stimulated progesterone synthesis on isolated bovine luteal cells. Endo. 103:1611-1618.

Wolfenson D, Inbar G, Roth Z, Kaim M, Bloch A, Braw R. 2004. Follicular dynamics and concentrations of steroids and gonadotropins in lactating cows and nulliparous heifers. Theriogenology. 62:1042-1055.

Vasenna R, Mapletoft RJ, Allodi S, Singh J, Adams GP. 2003. Morphology and developmental competence of bovine oocytes relative to follicular status. Theriogenology. 60:923-932.

Chitosan Nanoparticle of hCG (Human Chorionic Gonadotrophin) Hormone in Increasing Induction of Dairy Cattle Ovulation

Pamungkas FA, Sianturi RG, Wina E, Kusumingrum DA

[1]*Indonesian Research Institute for Animal Production, PO Box 221, Bogor 16002*
E-mail: fitrap@yahoo.com

(received 29-12-2015; revised 08-03-2016; accepted 25-03-2016)

ABSTRAK

Pamungkas FA, Sianturi RG, Wina E, Kusumaningrum DA. 2016. Nanopartikel chitosan hormon hCG (*Human Chorionic Gonadotrophin*) dalam meningkatkan induksi ovulasi pada sapi perah. JITV 21(1): 34-40. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1343

Suatu sistem rilis hormon hCG (human chorionic gonadotropin) yang terkontrol membantu mengatasi degradasi hormon hCG yang cepat dalam darah dan menghindari penggunaan injeksi hormon berkali-kali untuk tujuan induksi ovulasi serta meningkatkan efektifitas reproduksi. Penelitian bertujuan mempersiapkan nanopartikel chitosan hormon hCG (CS-NPh) serta mengetahui efektifitas penggunaannya melalui nasal spray. Paramater yang diamati meliputi karakteristik sifat fisikokimia CS-NPh dan pengamatan ukuran folikel, korpus luteum, waktu ovulasi dan onset berahi setelah pemberian CS-NPh secara nasal spray dibandingkan dengan hCG secara intramuskular (kontrol) dengan dosis 1.000 IU pada sapi perah. Hasil penelitian menunjukkan bahwa karakteristik sifat fisikokimia CS-NPh masih dalam rentang ukuran nanopartikel dengan distribusi massa molekul yang baik dan lebih stabil, sehingga dapat digunakan sebagai komponen pembawa hormon. Hasil penelitian diperoleh bahwa waktu ovulasi setelah pemberian hormon hCG secara intramuskular (hari ke 3,13±0,35) dan CS-NPh secara nasal spray (hari ke 3,33±0,49) dengan ukuran folikel yang diovulasikan berturut-turut sebesar 1,62±0,22 dan 1,76±0,28 cm tidak menunjukkan perbedaan yang nyata (p>0,05), begitu juga untuk ukuran korpus luteum dan onset berahi. Hal ini menunjukkan bahwa pemberian CS-NPh secara nasal spray dapat digunakan dalam meningkatkan induksi ovulasi pada sapi perah.

Kata Kunci: Nanopartikel, hCG, Nasal Spray, Ovulasi

ABSTRACT

Pamungkas FA, Sianturi RG, Wina E, Kusumaningrum DA. 2016. Chitosan nanoparticle of hCG (Human Chorionic Gonadotrophin) hormone in increasing induction of dairy cattle ovulation. JITV 21(1): 34-40. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1343

A controlled release delivery system of human chorionic gonadotropin (hCG) hormone helps to overcome the rapid degradation of hCG hormone in the blood, to avoid the use of multiple injections for ovulation induction and to enhance reproductive efficacy. This study aimed to prepare chitosan nanoparticles hCG (CS-NPh) and to determine its efficacy as nasal spray of CS-NPh. The observed parameters include physico-chemical characteristics of CS-NPh and the follicle size, corpus luteum, the time of ovulation and onset of estrus performed after administration of CS-NPh as a nasal spray compared with intramuscular hCG (control) at a dose of 1,000 IU in dairy cattles. The result showed that the formation of the hormone hCG nanoparticles is still in the size range of nanoparticles with a well and more stable molecular mass distribution, so it can be used as a carrier component of hormones. The result showed that the time of ovulation after hCG by intramuscular (day to 3.13±0.35) and CS-NPh as a nasal spray (days to 3.33±0.49) with the follicle size by 1.62±0.22 and 1.76±0.28 cm showed no significant differences (p> 0.05), likewise the size of the corpus luteum and onset of oestrus.. This indicates that administration of CS-NPh as a nasal spray can be used in enhancing the induction of ovulation in dairy cattles.

Key Words: Nanoparticles, hCG, Nasal Spray, Ovulation

INTRODUCTION

Estrus synchronization in dairy cattle is used for facilitating artificial insemination application, saving labor and time in detecting estrus. Estrus synchronization is one way of controlling estrus, to synchronize pre-ovulation condition, minimize variation of follicle maturity, luteolysis and increase estrus at the same time which around 20.5 days of estrus interval and ovulation (Cavalieri et al. 2004). In general, pregnancy rate of Artificial Insemination (AI) after an estrus synchronization in cattle was not as expected or not consistent due to varied of estrus and ovulation time determination due to long estrous cycle in cattle (Roelofs et al. 2005).

Induction of ovulation using Human Chorionic Gonadotropin (hCG) hormone injection together with estrus synchronization program enabled to determine accuracy of estrus and ovulation time which eventually gets improving animal fertility (Johnson et al. 2010).

Beside giving stress to animal, hCG has fast degradation of endopeptidase and exopeptidase in pituitary. Another approach is using slow release delivery system through implantation method. Hormone content packed or attached on matrix nanoparticle using biodegradable polymer has been used as potential hormone carrier by its capability to improve hormone stability and protected from rapid degradation during hormone transportation and controlling of bioactive agent releasing (Bhadra et al. 2002; Vila et al. 2002; Kommareddy et al. 2005; Rather et al. 2013).

The nasal route has received a great deal of attention as a convenient and reliable method for the systemic administration of drugs. Nasal delivery has been explored as an alternative administration route to target drugs directly to the brain via the olfactory neurons (Ulrika et al. 2005). Nasal mucociliary clearance is one of the most important limiting factors for nasal drug delivery. It severely limits the time allowed for drug absorption to occur and effectively rules out sustained nasal drug administration. However, bioadhesive polymers can be used to increase the nasal residence time, thus allowing longer absorption times, and to achieve a more intimate contact with the nasal mucosa, which results in a higher concentration gradient and subsequent increased absorption (Ugwoke et al. 2005).

Another important limiting factor in the nasal application is the low permeability of the nasal mucosa for the drugs. It seems to be necessary to consider an absorption enhancement mechanism for co-administration of drugs with either mucoadhesive polymers or penetration enhancers or combination of the two (Vasir et al. 2003). Chitosan nanoparticle may strongly bind negatively charged materials such as cell surface and mucosal fluid. Mucosal fluid contained mucin which had different chemical composition, however, some consisted of significant proportion from sialic acid. In physiological condition, sialic acid carried negative charge causing strong electrostatic interaction between mucin and chitosan in soluble (Dhawan et al. 2004).

The purposes of this study were to prepare chitosan nanoparticle of hCG hormone and to determine its effectivity through its use as nasal spray by observing the functional response such as follicle growth, ovulation time, and emergence of estrous signs.

MATERIALS AND METHODS

Preparation and characterization of chitosan nanoparticle of hCG hormone (CS-NPh) were conducted in the Reproduction Laboratory of the Indonesian Research Institute for Animal Production (IRIAP) and Nanotechnology Laboratory of Indonesian Center for Agricultural Post Harvest Research and Development (ICAPOSTRD). At the same time, *in vitro* examination was conducted in of the Animal House of IRIAP.

Formulation of chitosan nanoparticle of hCG hormone

Chemical materials used in formulation of chitosan nanoparticle of hCG were Chitosan (degree of deacetylation ≥75%) and sodium tripolyphosphate (TPP) produced by Sigma-Aldrich Corporation (St. Louis, MN), acetic acid (glacial) produced by Merck Millipore Corporation (Darmstadt, Jerman), hCG hormone (Choluron®) produced by Intervet/Merck Animal Health, Holland.

Chitosan nanoparticle of hCG hormone was arranged by ionic interaction process, in accordance with modified procedure performed by Wang et al. (2008). Two solution were prepared, namely 0.2% b/v of chitosan in 1% v/v acetat acid, and TPP (0.1% b/v) that diluted in aquadest. TPP (2.0 ml) solution was slowly added using syringe into acetic acid chitosan solution (5.0 ml) under 1500-rpm speed magnetic stirring until nanoparticle formed. Then hCG hormone (Choluron®) was added slowly into chitosan nanoparticle solution with concentration established was 500 IU/ml under 1500 rpm speed magnetic stirring for 10 minutes. As 1 N HCL or NaOH solution was added to manage suspension pH at 5.5.

Characteristics of physicochemical properties of chitosan nanoparticle of hCG hormone

Average size of particle and distribution were measured by photon correlation spectroscopy (PCS, dynamic light scattering, DLS) technique which is a multipurpose tool to estimate distribution of fine particle size of a material in range 0.6 nm – 7 µm. As 5 µm nanoconjugate suspension was diluted in 5 ml deionized water and be shaken slowly and then put into analyzer. After reaching expected intensity, analysis was conducted to obtain average particle size and polydispersity index (PDI) of the samples. Measurement of zeta potential was a way to know the stability of every coloid system which determined by electrophoretic light scattering (ELS).

Evaluation of entrapment efficiency of chitosan nanoparticle of hCG hormone

Entrapment efficiency (EE) process was determined by separating nanoparticle from suspension that consisting hCG non-entraped with ultra-centrifugation at 12.000 rpm, 10ºC for 10 minutes. The amount of free hCG hormone in supernatant was measured by spectrophotometry UV at λ = 260 nm (Katas et al.

2013). Entrapment efficiency (EE%) of hCG in CS-NPh was calculated according the following formula:

$$EE\ \% = \frac{\text{hCG amount} - \text{free hCG in supernatant}}{\text{Total amount of hCG}} \times 100$$

Estrus-ovulation synchronization

Cows used were 2-5 years old Friesian Holstein (FH), at least experienced once bear, non pregnant one, and had normal estrus cycle. They were ultrasound (USG) first, to monitor ovaries and examine their follicles and corpus luteum (CL) condition, Cows, then injected with Prostaglandin / $PGF_{2\alpha}$ (Lutalyse® 5-ml) intramuscularly (IM) and repeated at day 11. Then, they were divided into 2 groups as following:

Group A : Injected by 1.000 IU hCG hormone intramuscularly at day 2 after the last Prostaglandin ($PGF_{2\alpha}$) injection

Group B : Chitosan nanoparticle of hCG (CS-NPh) providing at 1.000 IU by nasal spray at day 2 after the last $PGF_{2\alpha}$ injection

Ultrasonography

USG was conducted to observe the ovarian dynamics, by measuring diameter of follicles and CL development. Diameter development pattern of follicles and CL were monitored using Ultrasonography (brand SIUI Veterinary model CST 7700V with 10.4 inchi monitor LCD and Linier Rectal Probe 7.5 MHz). USG observation was performed everyday started from second $PGF_{2\alpha}$ injection until ovulation. Before the transrectal USG observation, cow's feces were emptied and dragged first from the rectum. Scanning was conducted to right and left ovarium, and diameter of entire visible follicles and CL were measured one by one.

Estrus observation

Observation of estrus was conducted along with USG observation. It was focusedon physical changes of outside reproduction organs during estrus observation such as swelling of vulva, mucus discharged from vulva, reddening of vagina mucous, and it was warm if touched.

Data analysis

Data were analysed by Analysis of Variance (ANOVA) and then continued by Dunnet further testing. Data were processed by SPSS version 19.

RESULT AND DISCUSSION

Chitosan nanoparticle of hCG in this study was prepared according to ionic interaction process (ionic gelation), where the process of nanoparticle production depend on positively charged particles dissolved in chitosan molecule while pH had an important role during its production process. Characteristic of physicochemical properties of chitosan nanoparticle - hCG hormone was showed in Table 1.

Table 1. Characteristic of physiochemical properties of chitosan nanoparticle of hCG hormone

Parameters	Value
Particle size (nm)	226.17±26.27
Polydispersity index	0.301±0.03
Zeta potential (mV)	+21.22±1.51
entrapment efficiency (%)	54.11±12.94

Acidity of chitosan nanoparticle-hCG hormone formed was at initial pH 3.79±0.04 (not shown in the table) and adjusted to pH 5.5. Therefore, the uptake of nanoparticle close to neutral pH may prove useful in the controlled release of a drug from a pH-sensitive. Low pH is a well known sensory irritant in pathological conditions such as inflammation in the nasal mucosa. Acid exposure cause marked nasal irritation via a specific subset of sensory nerves (Dalton et al. 2010). Chitosan in acid media might interact with negatively charged TPP form inter and intra-moleculs producing crossed ionic-chitosan nanoparticle (De Campos et al. 2001; Xu & Du 2003). Mi et al. (2003) reported that TPP diluted in water separated hydroxide ion (OH-) and tripolyphosphoric (P3O105-and HP3O104) at pH 9. At basic pH, both hydroxide and tripolyphosphoric ions competed in ionic reaction with protoned-amino group (NH3+) from chitosan solution (pH 3.5) with deprotonation or crossed-ion. Availability of protoned-amino group caused crossed-ionic process easier for chitosan with high deacetilation rate (Agnihotri et al. 2004). Ionic gelation method, nanoparticle was produced by spontantenous formation from smaller size with positive charge (De Campos et al. 2001; Pan et al. 2002) at room themperature (Hu et al. 2002).

Nanoparticle formation by conditioning TPP at acid pH (<6) resulting low cutting level of phosphor chain and formation of nanoparticle by crossed-ion processing. Dudhani & Kosaraju (2010) reported that formation of chitosan nanoparticle at pH 4.5, reduced the size of particle and increasing zeta potential than the one formed at pH 5.5. Zengshuan et al. (2002) reported

that efficiency of insulin hormone interaction with chitosan nanoparticle at pH 5.3 was lower than at pH 6.1 but had stronger and more stable charge interaction in acidic and enzymatic hydrolysis condition.

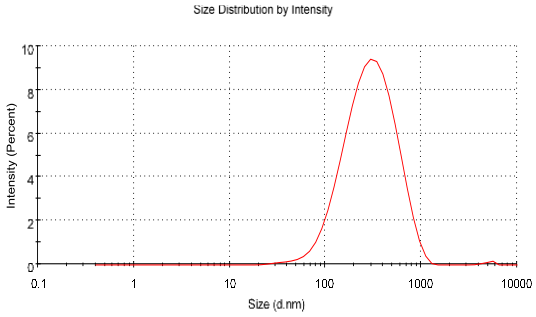

Figure 1. Particle size distributions of chitosan nanoparticles of hCG hormone

The distribution of particle size for chitosan nanoparticles hCG hormone is given in Tabel 1 and Figure 1. The results of this study showed that particle size of chitosan nanoparticle- hCG hormone was 226.17±26.27 nm and polydispersity index (PDI) value was 0.301±0.03. Particle size is the most important characteristic in nanoparticle system. Particle size in this study was in range with nanoparticle size reported by Mohanraj & Chen (2006) who said that nanoparticle was a dispersion or solid particle around 10-1000 nm size. As polydispersity index score was in size range of distribution of muleculer mass in certain sample. Result showed that PDI score was 0.3, where this score showed that calculation result of average molecule weight was divided by amount of average molecule weight. The closer to zero means that the distribution was getting better.

Average particle size in this study was higher than Zhang et al. (2004) who reported that low molecular weight chitosan with concentration by 0.1% (w/w) in nanoparticle formation resulting distribution of particle size by 153-500 nm. Dudhani & Kosaraju (2010) reported that formation of catechin chitosan nanoparticle at pH 5.5 resulted particle size as 130±5 nm. Rather et al. (2013) said that particle size of chitosan nanoparticle of Luteinizing Hormone Releasing Hormone (LHRH) at pH 6.5 was 114±10.3 nm with PDI score 0.335. In contrast with Zengshuan et al. (2002) who reported that formation of chitosan nanoparticle of insulin hormone at pH 5.3 resulting particle size by 243-271 nm with 0.50-0.57 of PDI score. Difference of average particle size and PDI score was due to difference in pH and the hormone used. Zhang *et al*. (2004) reported that manipulation of charged particle might be performed with varied pH but was followed by disintegration of particle due to weak interaction of chitosan and TPP (Zhang & Kosaraju 2007).

Surface charge is an important parameter to examine stability of suspention and adhesion of particular system in biological surface, so that evaluation of zeta potential should be an important part of nanoparticle. Zeta potential was a surface charge that might influence stability of particle in suspention due to electrostatic repulsion between particles (Qi et al. 2004). Zeta potential and entrapment efficiency of chitosan nanoparticle- hCG in this study were +21.22±1.51 mV and 54.11±12.94 %, respectively.

Analysis of zeta of chitosan nanoparticle-hCG in this study showed positive (+) sign and more than 20 mv. Positively charged-chitosan nanoparticle was a uniq characterization of chitosan/TPP particle. In a formation mechanism of particle, positively charged-amina was neutralized by its interaction with negative charge in triphosphaste molecule and recidual amino group affecting positive zeta potential (Honary & Zahir 2013). Higher zeta potency value in certain range indicated that chitosan nanoparticle formed was not stable. Wang et al. (2008) said that long amino chain group might prevent aggregation in nanoparticle.

hCG hormone was a glycoprotein hormone which able to efficiently (90-100%) absorb into polimer at around isoelectric pH (pI) of protein due to minimalization electrostatic repulsion which increasing of stability conformation and shorter specific surface wide of protein molecule (Sanchez et al. 2008). Relation of hCG hormone and chitosan might be encouraged by hydrophobic interaction between –H bond, however its association was relatively unstable due to pH change in acid range (Nielsen et al. 2001). Wang et al. (2008) reported that chitosan nanoparticle of estradiol hormone prepared by ionic glass method had zeta potential score and entrapment efficiency by +25.4 mV and 67% respectively. While, Rather et al. (2013) said that chitosan nanoparticle of Luetinizing hormone releasing hormone (LHRH), with same method resulted zeta potention score and entrapment efficiency by -33.14±6.67 mV and 69% respectively.

In vivo study was conducted to analyze the application method of chitosan nanoparticle-hCG hormone (CS-NPh) by nasal spray. Absorption in the nasal cavity was faster and there was a direct tract from the nasal cavity to central nerve system increasing hormone capacity selectively in certain area in brain (Wang et al. 2008). Many studies in experimental animal showed that hormone concentration in brain was higher after hormone application via nasal than via intravena injection that showing a tract from nasal to brain (Illum 2000; Vyas et al. 2005). Chitosan nanoparticle may strongly bind materials such as cell surface and mucus. Mucus consisted of mucin which has different chemical construction, however some of them consisted of different silate acid. At physiologic-pH, silate acid carried negative charge causing strong

interaction between mucin and chitosan in solution (Dhawan et al. 2004). Chitosan not only served to enhance adhesive between formulation and nasal tissue, but also acted in paracellular transportation process (Smith et al. 2004). Immunohistological studies have shown that chitosan can open the tight junctions between cells through an effect upon factin filaments. Unlike other absorption promoters, chitosan appears to be non-toxic and well tolerated by human subjects. This combination of bioadhesion and paracellular transport effects has led to a consideration of the use of chitosan for the delivery hormone via the nasal cavity (Wang et al. 2008).

Thirteenth of 43 synchronized-cows showed ovulation time before hCG providing, so that only 30 cows were included in the treatment group. This possibility due to cows already in luteal phase and already had CL after second $PGF_{2\alpha}$ injection, so that PGF2α acted to the CL. The lysis of CL decreased the progesterone level and causing loss barrier against gonadotropine hormone which induced follicle growth and maturation, estrus and ovulation. As said by Marietta et al. (2001) that $PGF_{2\alpha}$ hormone use for estrus syncrionization program was only effective if the animals already hada a CL. Then Santos et al. (2001) said that $PGF_{2\alpha}$ injection would decrease concentration of progesterone in blood so that ratio of concentration of estrogen and progesterone increased inducing animals showing estrus behaviour and then ovulation occured.

Ovulated follicle size and ovulation time after hCG injection intramuscularly and CS-NPh as a nasal spray showed in Table 2. Research result showed that hCG hormone intramuscularly injection and CS-NPh as a nasal spray did not show any significant difference (P>0.05) both in size of ovulated follicle and its ovulation time. Times of ovulation after hCG providing intramuscularly and CS-NPh as a nasal spray were at the day- 3.13±0.35 and 3.33±0.49 days, respectively with size of ovulated follicle was 1.62±0.22 and 1.76±0.28 cm, respectively. Time of ovulation related to the largest diameter follicle. Safilho et al. (2010) and Perry et al. (2005) reported that follicle ovulated in cattle, generally had diameter around 1.11-1.44 cm. Whereas, Cavalieri et al. (2004) reported that diameter of follicle ovulated in FH cattle was around 1.06-1.76 cm.

Beside follicle size observation, corpus luteum size and estrus onset observation after hCG applying were conducted and presented in Table 3. It shows that administration of hCG intramuscularly and CS-NPh by nasal spray did not show any significant difference (P>0/05) both in corpus luteum size or onset of estrus. The results of this research also showed that corpus luteum size decreased from day-0 to day-4 after the last PGF2α injection and hCG by IM (from 2.05±0.46 into 0.91±0.26 cm) and CS-NPh as a nasal spray (from 2.16±0.40 into 0.88±0.22 cm).

Principle of estrus synchronization using $PGF_{2\alpha}$ which act as luteolytic agent was regressing CL and causing loss of CL function and decline the

Table 2. Size of folicle ovulated and ovulation after hCG hormone providing

Group	n	Folicle size (cm)				Ovulation time (6th day)
		H0	H1	H2	Before ovulation	
A	15	1.22±0.20	1.40±0.20	1.49±0.21	1.62±0.22	3.13±0.35
B	15	1.25±0.25	1.42±0.28	1.56±0.28	1.76±0.28	3.33±0.49

Description: A : 1.000 IU hCG hormone providing intramuscularly
B : 1.000 IU CS-NPh providing by nasal spray
H0 : the last day of $PGF_{2\alpha}$ injection
H1 :-H2: day-1 to day-2 after the last $PGF_{2\alpha}$ injection

Table 3. Size of corpus lutheum and onset estrus after hCG hormone providing

Group	n	Size of corpus lutheum (cm)					Ovulation time (days)
		H0	H1	H2	H3	H4	
A	15	2.05±0.46	1.63±0.38	1.34±0.33	1.11±0.27	0.91±0.26	2.85±0.90
B	15	2.16±0.40	1.69±0.30	1.36±0.37	1.16±0.38	0.88±0.22	2.62±0.77

Description: A : 1.000 IU hCG hormone providing intramuscularly
B : 1.000 IU CS-NPh providing by nasal spray
H0 : the last day of $PGF_{2\alpha}$ hormone injection
H1-H2 : day-1 to day-2 after the last $PGF_{2\alpha}$ injection

progesterone concentration, against gonadotropine and there was a growth and maturation of follicle. Beside regressing CL, $PGF_{2\alpha}$ also decreased concentration of progesterone to less than 1.0 ng/ml within 24 hours (Yoshida & Nakao 2005). During estrus cycle, CL was an important structure in the term of size and duration. Appearance and disappearance of CL were responsible to estrus cycle (Hafez & Hafez 2001; Senger 2005). Estrus syncrhonization in animal was aimed to simultaneous their estrus in the same time. Research result showed that the emergence of estrus signs (swelling of vulva, mucus discharged from vulva, reddening of vagina mucousm, and it was warm if touched) after hCG providing intramuscularly and nasal spray was at the day- 2.85±0.90 and 2.62±0.77, respectively did not showing any significant difference (P>0.05).

CONCLUSION

Characteristic of chemical properties of particle size, polydispersity index, zeta potential and entrapment efficiency of chitosan nanoparticle of hCG hormone showed that nanoparticle formation of hCG hormone using chitosan polimer was still in nanoparticle size range and might be used as hormone carrier component.

Research result showed that time of ovulation after hCG providing intramuscularly (day- 3.13±0.35) and CS-NPh by nasal spray (day- 3.33±0.49) with size of follicles ovulated were 1.62±0.22 and 1.76±0.28 cm, respectively. Size of corpus luteum decreased from day-0 to day-4 after hCG providing intramuscularly (from 2.05±0.46 into 0.91±0.26 cm) or CS-NPh as a nasal spray (from 2.16±0.40 into 0.88±0.22 cm). As well as estrus onset and level of progesterone hormone after hCG providing intramuscularly did not show any significant difference (P>0.05) with CS-NPh as a nasal spray. This showed that CS-NPh providing as a nasal spray can be used in increasing induction of ovulation in dairy cattle.

ACKNOWLEDGEMENTS

This study was supported in part by DIPA from Indonesian Research Institute for Animal Production T.A. 2015 No. 1806.018.003/K-4/APBN 2015. The authors wish to thank M. Syaeri, Enok Mardiyah, Aqdi Faturahman Arrazy, Nia and Yanti for technical assistance and performing assays.

REFERENCES

Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. 2004. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Controlled Release. 100:5-28.

Bhadra D, Bhadra S, Jain P, Jain NK. 2002. Pegnology: a review of PEG-ylated systems. Pharmazie. 57:5-29.

Cavalieri J, Hepworth G, Macmillan KL. 2004. Ovarian follicular development in Holstein cows following synchronisation of oestrus with oestradiol benzoate and an intravaginal progesterone releasing insert for 5–9 days and duration of the oestrous cycle and concentrations of progesterone following ovulation. Anim Reprod Sci. 81:177-193.

Dalton PH, Opiekun RE, Gould M, McDermott R. Wilson T, Maute C, Ozdener MH, Zhao K, Emmett E, Lees PSJ, Herbert R, Moline J. 2010. Chemosensory loss: functional consequences of the world trade center disaster. Environ Health Perspect. 118:1251-1256.

De Campos AM, Sanchez A, Alonso MJ. 2001. Chitosan nanoparticles: A new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A. Int J Pharm. 224:159-168.

Dhawan S, Singla AK, Sinha VR. 2004. Evaluation of mucoadhesive properties of chitosan microspheres prepared by different methods. AAPS Pharm Sci Tech 5:122-128.

Dudhani AR, Kosaraju SL. 2010. Bioadhesive chitosan nanoparticles: Preparation and characterization. Carbohydrate Polymer. 81:243-251.

Hafez ESE, Hafez B. 2001. Reproduction in farm animals. 7th ed. Philadelphia (USA): Lippincot Williams & Wilkins.

Honary S, Zahir F. 2013. Effect of zeta potential on the properties of nano-drug delivery systems - a review (Part 1). Trop J Pharm Res. 12:255-264.

Hu Y, Jiang X, Ding Y, Ge H, Yuan Y, Yang C. 2002. Synthesis and characterization of chitosan–poly (acrylic acid) nanoparticles. Biomaterials. 23:3193-3201.

Illum L. 2000. Transport of drugs from the nasal cavity to the central nervous system, Eur J Pharm Sci. 11:1-18.

Johnson R, William AB, Evelin JC, Victor N, Yoonsung J, Melissa M. 2010. Roles of hCG in advancing follicular growth to ovulation after concurrent injections of PGF2α and GnRH in postpubertal Holstein heifers bearing a CL. Vet Medic Int. 2010:1-7.

Katas H, Raja MAG, Lam KL. 2013. Development of chitosan nanoparticles as a stable drug delivery system for protein/siRN. Int J Biomater 2013:1-9. doi: http://dx.doi.org/10.1155/2013/146320.

Kommareddy S, Tiwari SB, Amiji MM. 2005. Long-circulating polymeric nanovectors for tumor-selective gene delivery. Technol Cancer Res Treat. 4:615-625.

Marietta FW, Sayre B, Inskeep EK, Flores JA. 2001. Prostaglandin F2α Regulation of the Bovine Corpus Luteum Endothelin System during the Early and Midluteal Phase. Biol Reprod. 65:1710-1717.

Mi FL, Sung HW, Shyu SS, Su CC, Peng CK. 2003. Synthesis and characterization of biodegradable TPP/genipin co-crosslinked chitosan gel beads. Polymer. 44:6521-6530.

Mohanraj VJ, Chen Y. 2006. Nanoparticles - a review. Tropic J Pharm Res. 5:561-573.

Nielsen L, Khurana R, Coats A, Frokjaer S, Brange J, Vyas S, Uversky V, Fink AL. 2001. Effect of environmental factors on the kinetics of insulin fibril formation: Elucidation of the molecular mechanism. Biochemistry. 40:6036-6046.

Pan Y, Li Y, Zhao H, Zheng J, Xu H, Wei G. 2002. Bioadhesive polysaccharide in protein delivery system: Chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int J Pharm. 249:139-147.

Perry GA, Smith MF, Lucy MC, Green JA, Parks TE, Macneil MD, Roberts AJ, Geary TW. 2005. Relationship between follicle size at insemination and pregnancy success. Proc Natl Acad Sci. 102:5268-5273.

Qi L, Xu Z, Jiang X, Hu C, Zou X. 2004. Preparation and antibacterial activity of chitosan nanoparticles. Carbohydrate Res. 339:2693-2700.

Rather MA, Rupam S, Subodh G, Ferosekhan S, Ramya VL, Sanjay BJ. 2013. Chitosan-Nanoconjugated Hormone Nanoparticles for Sustained Surge of Gonadotropins and Enhanced Reproductive Output in Female Fish. Plos One. 8:1-11.

Roelofs JB, van Eerdenburg FJCM, Soede NM, Kemp. 2005. Various behavioral signs of estrous and their relationship with time of ovulation in dairy cattle. Theriogenology. 63:1366-1377.

Safilho MF, Crespilho AM, Santos JEP, Perry GA, Baruselli PS. 2010. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled Bos indicus cows. Anim Reprod. Sci. 120:23-30.

Sanchez S, Roldan M, Perez S, Fabregas E. 2008. Toward a fast, easy, and versatile immobilization of biomolecules into carbon nanotube/polysulfone-based biosensors for the detection of hCG hormone. Anal Chem. 80:6508-6514.

Santos JEP, Thatcher WW, Pool L, Ovrton MW. 2001. Effect of human chorionic gonadotropin on luteal function and reproductive performance of high producing lactating Holstein dairy cow. J Anim Sci. 79:2881-2894.

Senger PL. 2005. Pathways to Pregnancy and Parturition, 2nd ed. Moscow (Russia): Current conception Inc.

Smith J, Edward W, Michael D. 2004. Effect of Chitosan on Epithelial Cell Tight Junctions. Pharm Res. 21:43-49.

Ugwoke MI, Agu RU, Verbeke Nb, Kinget R. 2005. Nasal mucoadhesive drug delivery: Background, applications, trends and future perspectives. Adv Drug Deliv Rev. 57:1640-1665.

Ulrika W, P. Elena, J. Björn. 2005. Transfer of morphine along the olfactory pathway to the central nervous system after nasal administration to rodents. Eur J Pharm Sci. 24:565-573.

Vasir JK, Tambwekar K, Garg S. 2003. Bioadhesive microspheres as a controlled drug delivery system. Int J Pharm. 255:13-32.

Vila A, Sanchez A, Tobio M, Calvo P, Alonso MJ. 2002. Design of biodegradable particles for protein delivery. J Control Release. 78:15-24.

Vyas TK, Aliasgar S, Sudhanva M, Ambikanandan M. 2005. Intranasal drug delivery for brain targeting. Current Drug Delivery. 2:165-175.

Wang, X.M, N. Chi, X. Tang. 2008. Preparation of estradiol chitosan nanoparticles from improving nasal absorption and brain targeting. Eur J Pharma Biopharma. 70:735-740.

Xu Y, Du, Y. 2003. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. Int J Pharm. 250: 215-226.

Yoshida C, Nakao T. 2005. Response of plasma cortisol and progesterone after ACTH challenge in ovariectomized lactating dairy cows. J Reprod Dev. 51:99-107.

Zengshuan MA, Yeoh HH, Lee YL. 2002. Formulation pH modulates the interaction of insulin with chitosan nanoparticles. J Pharm Sci. 91: 1396-1404.

Zhang Q, X. Jiang, W. Jiang, W. Lu, L. Su, Z. Shi. 2004. Preparation of nimodipineloaded microemulsion for intranasal delivery and evaluation of the targeting efficiency to brain. Int J Pharm. 275:85-96.

Zhang H, Oh M, Allen C, Kumacheva E. 2004. Monodisperse chitosan nanoparticles for mucosal drug delivery. Biomacromolecules. 5:2461-2468.

Zhang L, Kosaraju SL. 2007. Biopolymeric delivery system for controlled release of polyphenolic antioxidants. Eur Polymer J. 43:2956-2966.

Phylogenetic Tree of Kuantan Cattle by DNA Barcoding

Hidayati[1,2], Misrianti R[1,2], Ali A[2]

[1]*Breeding and Genetic Laboratory, Agricultural and Animal Science Faculty*
[2]*Agricultural and Animal Science Faculty*
State Islamic University of Sultan Syarif Kasim - Riau
Kampus II Raja Ali Haji Jl. HR.Soebrantas Nomor 15 Simpang Baru Panam Pekanbaru 28293
E-mail: yati_suska@yahoo.com

(received 05-01-2016; revised 15-03-2016; accepted 21-03-2016)

ABSTRAK

Hidayati, Misrianti R, Ali A. 2016. Pohon filogenetik sapi Kuantan menggunakan DNA barcode. JITV 21(1): 41-48. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1351

Sapi kuantan merupakan breed sapi potong lokal Riau yang belum diketahui asal usulnya. Sapi kuantan banyak ditemukan di daerah Kabupaten Indargiri Hulu dan Kabupaten Kuantan Singingi. Berdasarkan tampilan fenotipe, sapi kuantan mirip dengan sapi pesisir (bangsa sapi potong lokal Sumatera Barat). Menurut beberapa peternak, asal-usul sapi kuantan merupakan sapi pesisir yang dibawa oleh perantau minang ke daerah ini. Tujuan penelitian ini untuk membuktikan apakah asal usul sapi kuantan merupakan sapi pesisir melalui analisis keragaman genetik menggunakan DNA barcode. DNA barcode yang digunakan adalah gene *Cytochrome oxidase sub unit I*, merupakan salah satu gene yang ditemukan pada DNA mitochondria. Isolasi DNA berhasil dilakukan pada 25 sampel darah sapi kuantan betina dewasa dan 18 sampel darah sapi pesisir betina dewasa. Amplifikasi ruas gene COI menggunakan metode *Polymerase Chain Reaction.* Primer forward yang digunakan dalam penelitian ini adalah F'5 TTCTCAACCAACCATAAAGATATTGG-3' dan primer reverse 5'-TAGACTTCGGGGTGTCCAAAGAATCA-3', mengapit ruas gene COI sapi kuantan dan sapi pesisir dari basa 5711 - 6420 (Genebank nomor akses NC_005971) sepanjang 710 bp. Hasil analisis sekuens menggunakan Program MEGA 5.2 menunjukkan bahwa ditemukan 6 titik polimorpik yang membentuk 7 haplotype pada sapi kuantan dan 9 titik polimorfik yang membentuk 12 haplotype pada sapi pesisir. Hasil analisis jarak genetik dan phon filogenetik menunjukkan bahwa sapi kuantan dan sapi pesisir berada pada kelompok yang sama dengan *Bos indicus*. Mutasi pada ruas gene COI sangat kecil dan belum dapat menjelaskan perbedaan diantara breed. Hasil penelitian ini juga menegaskan bahwa berdasarkan garis keturunan induk asal usul sapi kuatnan adalah dari *Bos indicus* sama seperti sapi pesisir.

Kata Kunci: Gene COI, Polimorfik, Sapi Kuantan, Jarak Genetik, Pohon Filogenetik

ABSTRACT

Hidayati, Misrianti R, Ali A. 2016. Phylogenetic tree of Kuantan cattle by DNA barcoding. JITV 21(1): 41-48. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1351

Kuantan cattle is one of local beef cattle breed of Riau Province which its origin was unknown. Kuantan cattle are commonly found in Indragiri Hulu and Kuantan Singingi Regency. Based on phenotype characterizations, kuantan cattles are similar with pesisir cattle (West Sumatera beef cattle). Historically, kuantan cattle were pesisir cattle brought by "minang" immigrants (Immigrant from West Sumatera) to this region. The purpose of this study was to analyze the origin of the kuantan cattle through genetic diversity analysis using DNA barcode. DNA barcode used was Cytochrome oxidase subunit I gene which was found in the mtDNA. DNA isolation was done on 25 kuantan's blood samples and 18 pesisir blood samples. Amplification of COI gene segment used Polymerase Chain Reaction technique. The forward primer sequence used in this study was F'5 TTCTCAACCAACCATAAAGATATTGG-3' and the reverse primer sequence used was reverse 5'-TAGACTTCGGGGTGTCCAAAGAATCA-3. It squeezed kuantan and pesisir sequence 5711 - 6420 base (GeneBank accession number NC_005971) with length by 710 bp. Analysis result of sequence using MEGA 5.2 Program showed that there were 6 polymorphic sites establishing 7 haplotypes on kuantan cattle and 9 polymorphic sites establishing 12 haplotypes on pesisir cattle. Based on genetic distance and phylogeney tree, kuantan and pesisir cattle were in same group with *Bos indicus*. Mutation in the COI gene segment in this study was too small and was not able to distinguish the difference of those breeds. The result of neighbor joining analyze indicated that kuantan cattle origin was from *Bos indicus* just like pesisir cattle.

Key Words: COI Gene, Polymorphic, Kuantan Cattle, Genetic Distance, Phylogenetic Tree

INTRODUCTION

Kuantan cattle, one of local beef cattle breed of Riau Province which was registered as No.1052/Kpts/S.R.120/10/2014 by Ministry of Agriculture on 14 October 2014, has been designated as one of Indonesian native cattle breed. Morphologically, kuantan bull had body length by 103.78 cm; chest girth by 126.22 cm; and shoulder height by 99.28 cm, while kuantan cow had body length

by 102.35 cm; chest girth 123.27 cm; and shoulder height 99.19 cm (Ministry of Agriculture Republic of Indonesia 2014). Kuantan cattle were found along the Kuantan River (from Kuantan Singingi Regency to Indragiri Hulu Regency). Total population of kuantan cattle in Indragiri Hulu Regency (5950 heads) was more than in Kuantan Singingi which were 2386 heads (Department of Animal Husbandry and Animal Health of Riau Province 2011). This kuantan cattle has been reared with extensive and semi intensive system for a long term.

Dominant color of kuantan cattle was white-brown with upward-curved horns and white legs. Phenotypically, kuantan cattle are similar with pesisir cattle and historically, kuantan cattle were pesisir cattle brought by "minang" immigrants (from West Sumatera) to this region. The development of every species under its particular natural ecosystem, environmental, and socio-economic conditions has led to each having its own specific genetic characteristics (Yang et al. 2013). Domestication of livestock species and a long history of migrations, selection and adaptation have created an enormous variety of breeds (Hailu & Getu 2015). The most domestic cattle in Indonesia belong to *Bos taurus* or *Bos indicus* while Indonesian Bali cattle was domesticated form banteng (*Bos javanicus*) (Kusdiantoro et al. 2009)

One of conventional methods applied in animal genetic resources assessment is morphological markers. Morphological markers, normally refer to external animal characteristics (animal's phenotype), which can be obtained by direct visual observation and measurement. Animal's phenotype is determined by its genetic, environment and interaction of both. Evolution of farm animal genetic resources through morphological markers based on subjective judgments and descriptions and the conclusions reached were often not completely accurate. Information on genetic diversity and origin of kuantan cattle has not been reported.

Determination of breed or line of livestock by Indonesian government was aimed to guarantee the utilization and conservation of animal genetic resources in a sustainable manner, as well as legal protection providing for breed or lines of the existing livestock. Management of animal genetic resources is essential for the achievement of global food security emphasizing global food production, improvement of productivity and food availability. People may use genetic resources to develop animal production to meet food requirement. However, sufficient genetic markers for evaluating population structure and other aspect of animal genetic resources require genetic diversity assessment. One method for population characterization was DNA barcoding (Yang et al. 2013; Hailu & Getu 2015). A DNA barcode was a short DNA sequence from a standardized region of the mitochondrial DNA gene, mitochondrial cytochrome c oxidase I (COI), used for species identification (Hebert et al. 2003).

COI gene was a gene that responsible for final step in phosphorylation before the establishment of adenosine triphosphate (ATP) (Sutrisno et al. 2013). COI gene had a conserve sequence of mitochondrial DNA on livestock (Mueller 2006) and might be used to analyze the origin of livestock (Wilson 2010) and was suitable for evolutionary studies (Lunt et al. 1996). The advantages of COI gene as a DNA barcode were (1) a relatively short length of the gene which was about 648 bp, (2) relatively stable and not prone to mutation, (3) variability was very low 1-2%, (4) much amount of copies was easily to be amplified than genes –gene nuclear DNA (Sutrisno et al. 2013).

Some research reports using DNA barcode on birds (Herbert et al. 2004), Indonesian local cattle (Febriana 2011), chicken (Gao et al. 2011), cetartiodactyla (Zein & Fitri 2012), bali cattle (Syed-Shabthar et al. 2013) and Indonesian local buffalo (Saputra et al. 2013) has been reported.

This study was aimed to obtain phylogenetic tree of kuantan cattle and to study whether pesisir cattle was the origin of the kuantan cattle based on DNA barcoding.

MATERIALS AND METHODS

Materials

Blood samples of more than two years old kuantan cow (n=25) from Kuantan Singingi (Riau Province) (Figure 1) and more than two years old pesisir cow (n=18) from central of superior cattle-forage breeding of Padang Mengatas, West Sumatera (Figure 2) were collected from jugular vein using 3 mL syringe. Those blood samples were preserved in EDTA and kept in room temperature from October to November 2015 for laboratory analysis.

DNA isolation

DNA isolation was done in Animal Molecular Genetic Laboratory, Bogor Agricultural University on November 2015. Genomic DNA was extracted using Phenol-chloroform Technique (Sambrook et al. 1989) and modified by buffer lysis cell use (250 µL 1 X STE, 40 µL SDS and 10 µL Proteinase-K). The DNA was purified by adding 40 µL 5M NaCl, 400 µL phenol chloroform and 400 µL CIAA (Chloroform Iso Amyl Alcohol) and precipitated using 40 µL 5M NaCl and 800 µL ethanol absolute. The precipitation was washed once by adding 800 µL of 70% ethanol and centrifuged at 12.000 rpm of speed for 5 minutes. Ethanol was discarded and evaporated. The precipitated DNA was dissolved in 100 µL of 80% TE (Elution buffer).

Figure 1. Kuantan cattle in Kuantan Singingi Regency, Riau Province.

Figure 2. Pesisir cattle in central of superior cattle-forage breeding of Padang Mengatas, Lima Puluh Kota, West Sumatra.

```
5581 tctgaatttg caattcaacg tgtaaattca ccacagagct tggtaaaaag aggagtcaaa
5641 cctctatctt tagatttaca gtctaatgct ttgctcagcc attttaccca tgttcattaa
5701 ccgctgacta ttctcaacca accataaaga tattggtacc ctttacctac tatttggtgc
5761 ttgggccggt atagtaggaa cagctttaag ccttctaatt cgcgctgaat taggccaacc
5821 cggaactctg ctcggagacg accaaatcta caacgtagtt gtaaccgcac acgcatttgt
5881 aataatcttc tttatagtaa taccaatcat aattggaggg ttcggtaact gacttgttcc
5941 cctaataatt ggtgctcccg atatagcatt tccccgaata aataatataa gcttctgact
6001 tctccctccc tcattcctac tactcctcgc atcctctata gttgaagctg gggcaggaac
6061 aggctgaacc gtgtaccctc ccttagcagg caacctagcc catgcaggag cttcagttga
6121 tctaaccatt ttctctttac acttagcagg agtttcctca attttaggag ccatcaactt
6181 cattacaaca attatcaaca taaagccccc cgcaatgtca caataccaaa cccctctatt
6241 cgtatgatcc gtaataatta ccgccgtact actactactc tcgctccctg tattagcagc
6301 cggcatcaca atgctattaa cagaccggaa cctaaatata actttcttcg acccggcagg
6361 aggaggagat cctattctat accaacactt attctgattc tttggacacc ccgaagtcta
6421 tattttaatc ttacctggat ttggaataat ctctcatatc gtaacctact actcaggaaa
```

Figure 3. BICOI amplicon sequence, Genebank NC_005971. The primers sites were underlined and bold.

Qualitative and quantitative test of DNA isolation

Qualitative test of DNA isolated was used on 1% agarose gel electrophoresis in 1XTAE solution. Electrophoresis was run at 100 volts for 35-40 minutes. Determination of concentration and purity level of the isolated DNA used spectrophotometry on 5 samples of the isolated DNA randomly.

DNA amplification and direct sequencing method

DNA was amplified using Polymerase Chain Reaction (PCR) technique carried out in Genetic and Breeding Laboratory, Faculty of Agricultural and Animal Science, Islamic State University of Sultan Syarif Kasim Riau on November 2015 - January 2016. Each PCR reaction was made with cocktail 50 ng (2-3 µL) DNA templates, 0.25 µM forward and reverse primer, 12.5 µL Dream Tag Green Master Mix from Thermo Scientific #K1081 and dH2O up to 25 µL. The forward primer sequence was F'5 TTCTCAACCAACCATAAAGATATTGG-3' and reverse primer sequence was reverse 5'-TAGACTTCGGGGTGTCCAAAGAATCA-3', in accordance with Febriani (2013). Position of forward and reverse primer in PCR product of COI gene was shown in Figure 3. Samples were initially denaturized at 93°C for 3 minutes and followed by 35-36 cycles of denaturation at 93°C for 30 second, annealing at 60°C for 30 second and extension at 72°C for 30 second. Final extension was at 72°C for 5 minutes. Master Cycler Personal 22331 Eppendorf was used for PCR amplification. PCR products were then separated on 1.5% agarose/1 x TAE, stained with 2.5 µL of ethidhium bromide (EtBr) and calibrated with 100 bp ladder marker. Electrophoresis chamber was run on 100

volt power supply for 35 minutes. Finally, the gel was visualized under Gel Doc. Forty three PCR product samples were then subjected to direct sequence (forward and reverse) analysis by dideoxy sequencing in ABI 3730 XL automated DNA sequencer at the First base laboratory Malaysia via commercial service on January 2016.

Data analysis

Results of sequences fragment of COI gene were analyzed with BioEdit (Hall 2011), MEGA version 5.2 software (Tamura et al. 2011). BLAST sequence was obtained from Gene Bank with accession number of KF 952276, KF 771228, KF 952284, KF 952285, HQ860420.1, HM 102290.1, KF771228.1, FJ 958333 and JX 218056.1. Method of neighboor-joining with kimura 2-parameter model was applied for reconstructing phylogeney tree.

RESULT AND DISCUSSION

Quality of DNA isolated and PCR amplification of COI gene

Qualitative method for identification, separation and purification of DNA fragments was by agarose gel electrophoresis (Fachtiyah, et al. 2011). DNA which might migrate on agarose gel or polyacrylamide gel was placed in a buffer solution which electrified in chamber. Negatively DNA will be moving positive direction. The success of DNA isolation characterized by a single band, bright and cleared in each well (Figure 4).

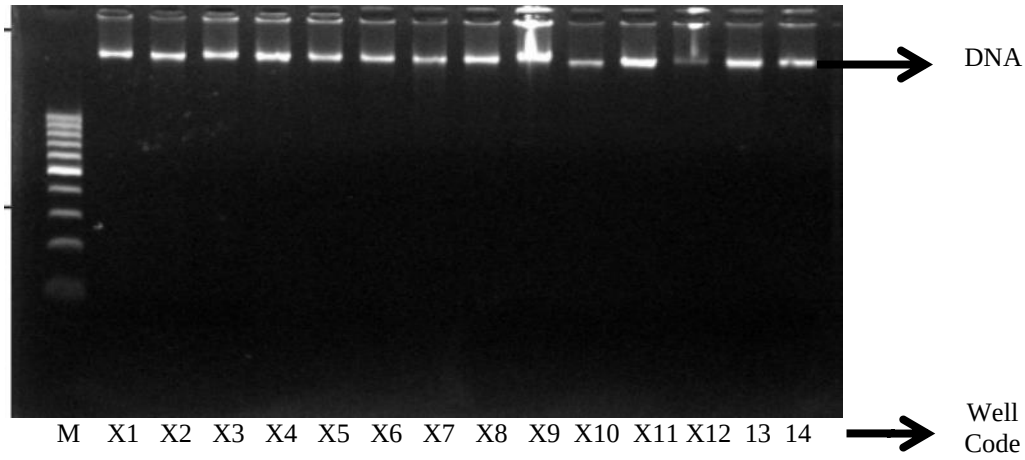

Figure 4. Qualitative test of DNA isolated.

Table 1. Concentration and purity level of isolated DNA

Sample code	DNA concentration (ng/µL)	Å 260	Å280	Purity level
Blanco	0.80	0.017	0.021	0.8
Kuantan cattle 19	89.40	1.79	0.99	1.80
Kuantan cattle 23	77.55	1.55	0.86	1.80
Kuantan cattle 30	121.45	2.43	1.34	1.81
Kuantan cattle X7	44.20	0.88	0.51	1.75
Kuantan cattle X12	27.45	0.55	0.33	1.64
Pesisir cattle 4796	97.45	1.95	1.08	1.80
Pesisir cattle 4848	49.30	0.99	0.56	1.77
Pesisir cattle 4856	66.10	1.32	0.75	1.77
Pesisir cattle 4857	29.95	0.60	0.35	1.71
Pesisir cattle 4859	84.85	1.70	0.95	1.79

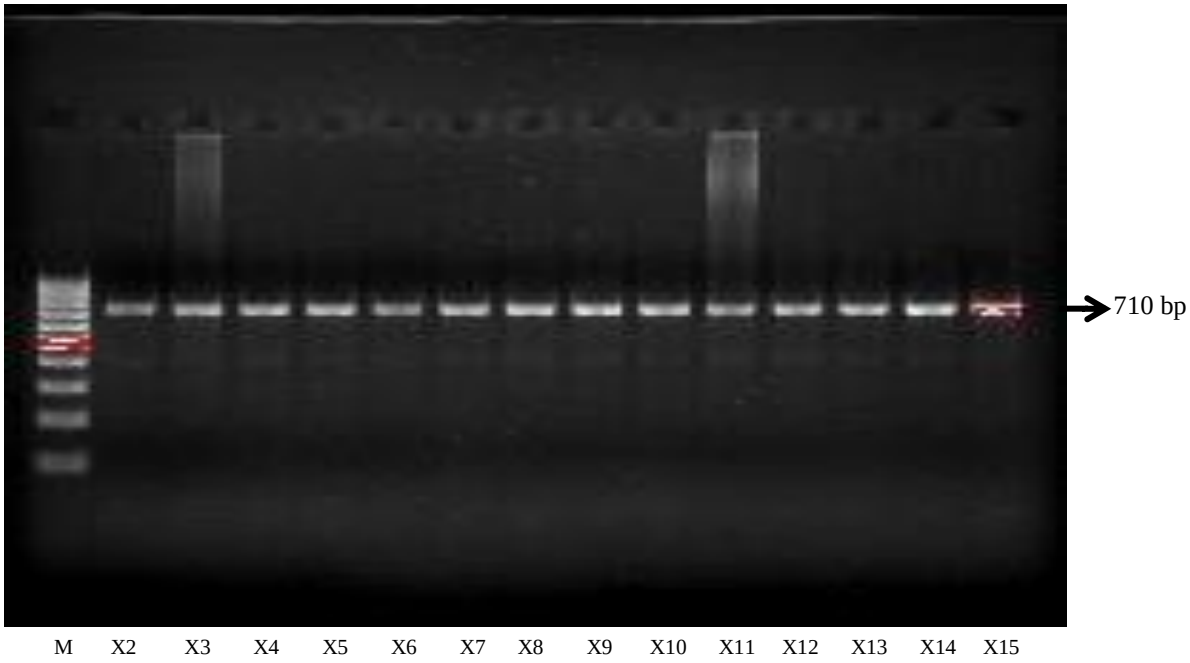

Figure 5. PCR amplification product of COI Gene (710 bp); M = DNA ladder 100 bp, X2, X3..Xn = samples codes.

Concentration of isolated kuantan's DNA was 27.45-121.45 ng/µL with a purity level of 1.64 to 1.81 (Table 1). DNA concentration of pesisir cattle was 29.9597.45 ng/µL with purity level of 1.71 to 1.80. Good purity levels of DNA isolated were 1.8 to 2.0. If the value exceeds of 2.0, the solution tested was still contaminated by membrane protein or other compounds so that the levels of plasmid DNA obtained was not pure. If it was less than 1.8, it means that too much ddH_2O was taken while DNA taken was too slightly. The success of PCR process was determined by accurately and level of concentration of DNA used as a template. The range of concentrations of DNA used in the PCR process was 25-50 ng/mL.

Primer has amplified COI gene of kuantan cattle and pesisir cattle successfully. It was indicated by the appearance of a single band at 710 bp (Figure 5). Annealing temperature was 60^0C for 45 seconds.

Diversity of COI gene on Kuantan and Pesisir cattle

Alignment of 25 kuantan cattle sequences showed six point mutations (c.9, c.25, c.35, c.60, c.111, c.617) forming seven haplotypes (A, B, C, D, E, F and G) (Table 2). The A haplotype was dominant (76%, n=19) on Kuantan cattle. The B (n=X2), C (X5); D (15); E (19); F (X7) and G haplotype (X) of kuantan cattle were detected 4% separately. On pesisir cattle it showed nine point mutations (c.7, c.16, c.35, c.42, c.67, c.74, c.145, c.254 and c.659) forming twelve haplotypes (A, B, C, D, E, F, G, H, I, J, K and L) (Table 2). The A haplotype on kuantan cattle and pesisir cattle was exactly similar. The A haplotype on kuantan cattle was the highest proportion (76%) on population than the B and J haplotypes on pesisir cattle (16.67%).

Genetic distance and phylogeny tree of COI gene on Kuantan and Pesisir cattle

Results of the analysis of genetic distance showed that kuantan and pesisir cattle might be grouped into *Bos indicus* because it had a genetic distance of 0.000 in all haplotypes, except on K Haplotype on pesisir cattle (0.002). Genetic distance of kuantan cattle and *Bos taurus* was in the range of 0.10 to 0.12. This result explains that based on the DNA barcode, kuantan cattle is in the same cluster with *Bos indicus* as well as pesisir cattle, aceh cattle and PO cattle. Cattle breeds have been developed in different ways depending on regional climates, nutritional conditions and selection for different purposes. Genetic drift also contributed in the process of breed differentiation. In very closely related breeds, the number of mutations cannot explain the observed genetic variation even when highly mutable DNA sequences are used.

The result was in accordance with Mitchell et al. (2010) who said that the genetic diversity of COI gene in the Bovidae was between 0.0 -1.92% (average 0.245) and 9.77% in average between the species. Genetic distance in the Bos, Capra, and Ovis genus was 3.5%, 0.0%, 0.003% respectively. Genetic distance between javanicus - indicus and javanicus - taurus species was 0.066% and 0.014%, respectively (Zein and Fitri, 2012). In this study, genetic distance between kuantan

and *Bos indicus* cattle was 0.00 and between kuantan and *Bos Taurus* was among 0.10-0.12. Genetic distance in height of COI gene between the species was low. It showed that COI gene was effective for identification at the level of species, genus and family and was appropriate to be used as a DNA barcode. For small size (N=50) population and mutation rate of $\beta = 10^{-3}$, the difference between computed inbreeding value assumed that mutation was small (less than 7 % of true value) and mutations might be neglected during 200 generations (Laval et al. 2002).

Reconstruction of phylogeny tree of kuantan cattle used neighboor-joining method. The result showed that all samples of cattle kuantan were in the same cluster with the *Bos indicus* with gene bank access number of KF 952 285, KF 952 284, KF 771 228 and *Bos Taurus* with gene bank access numbers of JX 18056.1. It was vary from *Bos Taurus* access number of HQ 860420.1, HM 102290.1, KF 771228.1 and FJ 958 333 with the genetic distance of 0.001 (Figure 6). This research showed that based on the maternal line, the ancestor of kuantan cattle was *Bos indicus* as well as pesisir cattle.

Table 2. Type of haplotype of Kuantan and Pesisir cattle

Haplotype type	Point mutation														n (%)
	7	9	16	25	35	42	60	67	74	111	145	254	617	659	
Kuantan cattle															
A Haplotype	A	G	G	A	T	T	G	A	T	-	G	C	A	C	19(76%)
B Haplotype	A	G	G	-	-	T	G	A	T	-	G	C	A	C	1(4%)
C Haplotype	A	G	G	-	T	T	G	A	T	T	G	C	A	C	1(4%)
D Haplotype	A	R	G	A	T	T	G	A	T	-	G	C	A	C	1(4%)
E Haplotype	A	G	G	A	-	T	G	A	T	-	G	C	A	C	1(4%)
F Haplotype	A	G	G	-	-	T	-	A	T	-	G	C	A	C	1(4%)
G Haplotype	A	G	G	-	T	T	G	A	T	-	G	C	-	C	1(4%)
Pesisir Cattle															
A Haplotype	A	G	G	A	T	T	G	A	T	-	G	C	A	C	1(5.56%)
B Haplotype	C	G	G	A	T	T	G	-	T	-	G	C	A	C	3(16.67%)
C Haplotype	A	G	G	A	-	-	G	A	T	-	G	C	A	C	1(5.56%)
D Haplotype	A	G	R	A	T	T	G	A	T	-	G	C	A	Y	1(5.56%)
E Haplotype	A	G	R	A	T	T	G	A	T	-	G	C	A	C	1(5.56%)
F Haplotype	A	G	G	A	T	T	G	A	-	-	G	M	A	C	1(5.56%)
G Haplotype	A	G	G	A	-	-	G	A	T	-	G	M	A	C	1(5.56%)
H Haplotype	A	G	G	A	-	-	G	A	-	-	G	M	A	C	1(5.56%)
I Haplotype	A	G	G	A	-	-	G	A	T	-	G	C	A	C	2(11.11%)
J Haplotype	A	G	G	A	T	T	G	A	T	-	G	C	A	C	3(16.67%)
K Haplotype	A	G	G	A	T	T	G	A	T	-	T	C	A	C	1(5.56%)
L Haplotype_	-	G	G	A	T	T	G	A	T	-	G	C	A	C	2(11.11%)

Figure 6. Phylogeny tree of Kuantan cattle by *Neighboor-Joining* Method.

CONCLUSION

Results of sequence analysis using the program MEGA 5.2 showed six point mutations which established 7 haplotypes on kuantan cattle. Nine point mutations on pesisir cattle established 12 haplotypes. Based on genetic distance, kuantan and pesisir cattle were in same group with *Bos indicus*. Mutations in the COI gene segment of kuantan and pesisir cattle in this study were too small and the difference cannot be clearly explained. The results of neighbor joining analysis indicated that the origin of the kuantan cattle was *Bos indicus* based on maternal lineage.

ACKNOWLEDGEMENT

Thanks to the Director of Islamic Higher Education, for providing a financial support through The Collective Competitive Research Ministry of Religious Affairs in 2015 with contract number Reg. PST/86/2015.

REFERENCES

Department of Animal Husbandry and Animal Health Riau Province. 2011. Laporan tahunan dinas peternakan dan kesehatan hewan provinsi Riau. Pekanbaru (Indones): Department of Animal Husbandry and Animal Health Riau Province.

Fachtiyah, Rumingtyas ELA, Widyarti S, Rahayu S. 2011. Biologi molekular. Jakarta (Indones): Erlangga.

Febriana A. 2011. Filogenei berdasarkan sekuens DNA Mitokondria gene Cytochrome oxidase I (gene COI) pada beberapa bangsa sapi lokal Indonesia (Skripsi). [Bogor (Indones)]: Institut Pertanian Bogor.

Gao YS, Tu YJ, Lu JX, Zhang XY. 2011. Studies on the DNA barcoding of two newly discovered chiecken breeds by mtDNA COI gene. J Anim Vet Adv. 10:1711-1713. doi: 10.3923/javaa.2011.1711.1713.

Hailu A, Getu A. 2015. Breed characterization tools and their applications. Int J Genet. 5:7-14. doi: 10.5829/idosi.ijg. 2015.5.1.92187

Hall T. 2011. BioEdit: An important software for molecular biology. GERF Bull Biosci. 2:60-61.

Hartl DL, Clark Ag. 1997. Principles of population genetic. Massachusetts (USA): Sinauer Associates Inc.

Hebert PDN, Cywinska A, Ball SL, de Waard JL. 2003. Biological identification through DNA barcodes. Philos Trans Ser B. 270:313-321. doi: 10.1098/rspb.2002. 2218.321.

Kusdiantoro M, Olsson M, van Tol HTA, Mikko S, Vlamings BH, Andersson G, Rodriguez-Martinez H, Purwantara B, Paling RW, Colenbrander B, Lenstra JA. 2009. On the origin of Indonesian cattle. PLos One. 4:1-6.

Laval G, San Cristobal M, Chevalet C. 2002. Measuring genetic distance between breeds: Use of same distances in various short term evolution models. Genet Sel Evo. 34:481-507. doi: 10.1051/gse:2002019.

Li W, de Graur D. 1991. Fundamental of molecular evolution. Sunderland (UK): Sinauer Associates, Inc.

Lunt DH, Zhang DS, Zhimura DM, Dewit GM. 1996. The insect cytochrome oxidase I gene: evolutionary pattern and conserve primer for phylogenetics studies. Insect Mol Biol. 5:153-165. doi: 10.1111/j.1365-2583.1996.tb00049.x.

Mitchell JE, Greta LM, Sergion OK, Mathew SL, Andrew PM, George A. 2010. Barcoding busmeat: Molecular identification of Central African and South American Harvested Vertebrates. Conserv Genet. 11:1389-1404.

Mueller RL. 2006. Evolutionary rates, divergenece dates, and the performance of mitochondrial genes in Bayesian phylogenetic analysis. Syst Biol. 55:289-300. doi: 10.1080/10635150500541672.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Clonning: A laboratory manual. 2nd ed. USA: Cold Spring Harbor Laboratory Press.

Saputra F, Jakaria, Sumantri C. 2013. Genetic variation of mtDNA cytochorome oxidase sub unit I (COI) in local swamp buffaloes in Indonesia. Media Peternakan. 36:165-170. doi: 10.5398/medpet.2013.36. 3.165.

Sutrisno H, Zein MSA, Sulandari S. 2013. DNA barcode. In: Zein MSA, Prawiradilaga DM, editors. DNA barcode fauna Indonesia. Jakarta (Indones): Kencana.

Syed-Shabthar SM, Rosli MK, Mohd-Zin NA, Romaino SM, Fazly-Ann ZA, Mahani MC, Abas-Mazni O, Zainuddin R, Yaakop S, Md-Zain BM. 2013. The moleculer phylogenetic signature of Bali cattle revealed by maternal and paternal markers. Mol Biol Rep. doi: 10.1007/s11033-013-2619-y.

Taberlet P. 1996. The use of mitochondrial DNA control region sequencing in conservation genetics. In: Smith TB, Wayne RK, editors. Molecular genetic approaches in conservation. New York (USA): Oxford University Press. p. 125-142.

Tamura K, Dudley J, Nei M, Kumar S. 2011. MEGA software (version 5): Molecular evolutionary genetics analysis. Center of Evolutionary Functional Geneomics Biodesign Institute. Arizona State University.

Wilson JJ. 2010. Assessing the value of DNA barcodes and other priority gene regions for molecular phylogenetics of Lepidoptera. PLos One. 5:e10525. doi: 10.1371/journal.pone.0010525.

Yang W, Kang X, Yang Q, Lin Y, Fang M. 2013. Review on the development of genotyping methods for assessing farm animal diversity. J Anim Sci Biotech. 4:1-6.

Yuwono T. 2006. Teori dan aplikasi polymerase chain reaction. Yogyakarta (Indones): Andi.

Zein MSA, Fitriana YS. 2012. Teknik molekuler untuk identifikasi species ordo *cetartiodactyla* menggunakan DNA barcode. Zoo Indonesia. 21:1-8.

Relationship of Extender and Packaging System an the Length of Preservation and the Quality of Chilled Semen of Boer Goat

Febretrisiana A, Anwar, Sinulingga S

Indonesian Goat Research Station, PO Box I Sei Putih, Galang 20585, North Sumatra

(received 22-01-2016; revised 08-03-2016; accepted 16-03-2016)

ABSTRAK

Febretrisiana A, Anwar, Sinulingga S. 2016. Hubungan jenis pengencer dan sistem pengemasan terhadap lama simpan dan kualitas semen cair kambing Boer. JITV 21(1): 49-54. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1350

Tujuan penelitian ini adalah untuk membandingkan penggunaan bahan pengencer yang berbeda (Triladyl dan Tris kuning telur (TKT)) serta pengaruh cara pengemasan semen cair (pool dan straw) terhadap kualitas semen cair kambing Boer. Semen ditampung menggunakan vagina buatan yang berasal tiga ekor kambing pejantan Boer berumur 2 tahun dengan bobot hidup 50-55 kg. Semen dievaluasi kemudian diencerkan dengan menggunakan pengencer Triladyl dan TKT kemudian disimpan di lemari pendingin dengan suhu 5^0C dalam kemasan yang berbeda yaitu dalam bentuk pool dan straw. Evaluasi dilakukan setiap hari selama 5 hari. Hasil penelitian menunjukkan motilitas sperma yang lebih baik diperoleh dengan menggunakan pengencer Triladyl dan dengan sistem pengemasan pool, lebih tinggi (P<0,05) bila dibandingkan menggunakan sistem pengemasana straw dan lebih tinggi bila menggunakan pengencer TKT dengan kedua sistem penyimpanan (masing-masing 45,8%, 26,1%, 32,1% dan 9,1%). Hal sama juga terlihat pada integritas membran spermatozoa yang terlihat lebih tinggi (P<0.05) bila menggunakan pengencer Triladyl baik dengan sistem penyimpanan pool (75,2%) maupun dengan straw (77,2%). Viabilitas spermatozoa dengan menggunakan pengencer Triladyl baik dengan pengemasan pool maupun straw mulai mengalami penurunan setelah 3 hari penyimpanan (77,1% dan 76,2%; P<0,05) sedangkan dengan pengencer TKT viabilitas spermatozoa menurun setelah 4 hari penyimpanan (73,2% dan 58,0%; P<0,05). Kesimpulan dari penelitian ini adalah kualitas semen cair kambing Boer menurun seiring dengan bertambahnya waktu penyimpanan dan pengencer Triladyl dengan sistem pengemasan pool menjadi metode terbaik untuk preservasi semen cair kambing Boer.

Kata Kunci: Semen Cair, Boer, Triladyl, Tris Kuning Telur, *Straw*

ABSTRACT

Febretrisiana A, Anwar, Sinulingga S. 2016. Relationship of extender and packaging system an the length of preservation and the quality of chilled semen of Boer goat. JITV 21(1): 49-54. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1350

The aim of this research was to compare the effectiveness of different extender (either Triladyl or Tris Egg Yolk extender) and different packaging method (pool and straw) of chilled semen an the length of preservation and the quality of chilled semen of Boer goat. Semen was collected using an artificial vagina from 3 two years old Boer bucks with body weight of 50-55 kg. It was evaluated under a microscope, then each was diluted either in Tris egg yolk extender (TEY) or Triladyl. Those diluted sperms were then packed either in pool or straw and preserved at 5^0C refrigerator. Sperm motility, viability and membrane integrity of each group were evaluated every 24 h for up to 5 days. Results showed that sperm motility in Triladyl of pool packaging system up to 3 days was higher than straw packaging system or TEY in pool or straw packaging system which were 45.8%, 26.1%, 32.1% and 9.1%, respectively (P<0.05). Percentage of sperm membrane integrity showed the same pattern to Triladyl both in pool and straw packaging system which was higher than TEY group (75.2% and 77,2%; P<0.05). Sperm viability in Triladyl both in pool or straw packaging system decreased (P<0.05) after 3 days of preservation (77.1% and 76.2%) but TEY significanly decreased after 4 days of preservation either in pool or straw packaging system (73.2% and 58.0%; P<0.05). It was concluded that sperm quality decreased with increasing of the length of preservation while Triladyl extender in pool packaging system showed the best quality.

Key Words: Chilled Semen, Boer, Triladyl, Tris Egg Yolk, Straw

INTRODUCTION

Artificial insemination program is one of reproduction technologies aimed to increase pregnancy rate in animal. It encourages crossing program of selected buck. The artificial insemination is used intensively in goat breeding system, especially in crossbreeding program to increase meat or milk yield and litter size. It was also used to optimize selection program and facilitate to control time of birth (Leboeuf et al. 2000). Generally, artificial insemination use frozen semen. Limited liquid nitrogen due to distribution or evaporation is an issue in frozen semen use. Limited container availability and late in reporting

estrus also affect the artificial insemination performance using frozen semen. Chilled semen use in artificial insemination became an alternative to anticipate those issues (Leethongdee 2010).

Preservation process of chilled semen was commonly performed at 4-5°C with limited shelf life by 24-48 h after collection (Mara 2007). Extender material is one of important factors determining success of chilled semen preservation. Extender material is materials added to assure physical and chemical requirement for spermatozoa, raise the volume, and protect the spermatozoa from a cold shock. Extender material could be used as nutrient resource of spermatozoa, preventing growth of germs and maintaining osmotic tension and electrolyte balance to keep spermatozoa alive (Ismaya 2014). Extender of semen should contain similar contents with physical and chemical properties of plasma semen, did not contain toxic substance, and did not restrict fertility ability of spermatozoa (Verberckmoes et al. 2004).

Environment also affects chilled semen quality of goat preserved at 5°C in long term. In oxygenated environment, spermatozoa have shorter vitality. This is related to function of the oxygen as oxidative element in metabolic producing harmful waste product of metabolic oxidation such as hydrogen peroxide. Salomon & Maxwell (2000) reported that lipid peroxide played a key role in aging process; decreasing spermatozoa vitality; inducing structure change especially in acrosome area; damage in spermatozoa structure; biochemical and functional damage including decrease of spermatozoa motility, membrane integrity, or fertilization ability.

This study was aimed to evaluate effect of preservation of chilled semen of Boer goat using different extender and preservation method against quality and vitality of spermatozoa preserved at 5°C. This study results were expected to solve technical issues in the field, simplifying and accelerating success of artificial insemination in goat in Indonesia.

MATERIALS AND METHODS

This study was conducted in Laboratory of Reproduction of Indonesian Goat Research Station, Sei Putih, North Sumatera. This study was conducted for 6 months (February-July 2015).

Sample of animal

In this study three two year old Boer bucks with body weight of 50-55 kg were used. Cage system and diet provision were conducted individually and with ad libitum of water.

Semen collection, process, and sperm preservation

Fresh semen was evaluated in laboratory. Semen dilution used two different extenders, Triladyl (commercial) and Tris Egg Yolk (TEY) with composition of aminomethane by 2.96 mL, yolk by 2 mL, citrate acid by 1.65 g, lactose by 2.16 mL, glycerol by 6 mL, penicillin and streptomycin by 1000 IU/mL, aquabidest ad by 100 mL. Concentration of final spermatozoa was 200 × 106/ml. Semen was preserved in 5°C refrigerator in 15 mL tube (pool) and 0.25mL straw. Examination of vitality of chilled semen by calculating percentage of motility, viability, and membrane integrity in day-0, 1, 2, 3, 4, and 5 of preservation.

Evaluation of motility

Percentage of spermatozoa motility was determined by observation of progresively moved soermatozoa conducted subjectively in 6 different points of view. Score provided was from 0% (there was no spermatozoa moved) to 100% (all spermatozoa moved forward).

Evaluation of membrane integrity

Membrane integrity of spermatozoa was evaluated using Hypoosmotic Swelling Test (HOS-Test) in 5 points of view randomally with circular tail spermatozoa (whole membrane plasma) or straigh tail spermatozoa (incomplete membrane plasma). Total number of spermatozoa calculated was 200 spermatozoa.

Evaluation of viability

Percentage of viability was percentage of life and death spermatozoa using eosin-negrosin staining method. Spermatozoa was cathegorized as life if the head was colorless and cathegorized as death if the head was red. Percentage of viability of spermatozoa was determined according to comparison of the number of life spermatozoa and total amount of spermatozoa. Total amount of spermatozoa counted was 200 heads.

Data analysis

Data of viability and quality of chilled semen preserved at 5°C in the two groups were in percentage and analyzed using analysis of variance (ANOVA). This study was in completely randomized design with 5 replications. The analysist was continued by Duncan's Multiple Range Test (DMRT) if there was a difference between the groups.

RESULT AND DISCUSSION

Sperm motility

Result showed that use of Trilady extender with pool packaging showed higher motility rate (P<0.05) up to day-5 of preservation than other groups (77.1%, 64.6%, 55.3%, 45.8%, 33.2% and 15.3% respectively at day 1, 2, 3, 4 and 5 of preservation) showed in Figure 1. Straw packaging of Trilady extender significantly (P<0.05) decreased motility rate since the first day of preservation (52.4%) to day-2 preservation (33.6%). Use of TEY extender showed lower motility both with pool and straw packaging system. Each only could maintain the quality of spermatozoa up to day-2 of preservation (41.9%). Quality of spermatozoa in day-1 of preservation was 49.3% (P<0.05). Use of Triladyl with pool packaging in day-3 of preservation showed feasible percentage for the artificial insemination.

It was showed that Triladyl extender with pool packaging system was able to maintain spermatozoa motility up to day-3 of preservation. Motility rate in all groups decreased along with increase of preservation time. Decrease of motility was assumed due to damage of spermatozoa during the preservation at low temperature (5°C). Chilling was one way to slow down cell metabolism to maintain cell viability. It was also able to decrease oxygen requirement and slow down acid accumulation as a result of apoptosis. Molecular activities and ion mobility regulated by thermal energy were basic of biological and chemical process. Decrease in thermometer slowed down the molecule activities. Biochemical process could not be separated from interaction process between molecules in catalyst reactions by enzyme and chilling method strongly influenced all those reaction components (Taylor 2006).

Addition of extender might protect spermatozoa from cold stock. Tris Egg Yolk was able to protect spermatozoa from cold sock, so that was used as basic material of extender (Alves et al. 2013). Low density lipoprotein (LDL) in Tris Egg Yolk consisted of 79% lipid and 21% protein with the main component was cholesterol. Structure of lipoprotein in Tris Egg Yolk was similar with structure of plasma membrane and it could protect the spermatozoa (Botham & Mayes 2009). However, in this study, Triladyl use showed better result than TEY. Fructose component in Triladyl extender played a role to maintain spermatozoa motility during preservation compared to lactose component in TEY extender. Other indication was caused by different energy use path in cell of each sugar was different related to metabolism of nitrogen (Medrano 2006). Fructose was included in to easily changed sugar in to energy source (Schorin 2012). Addition of fructose might be energy source for spermatozoa as known it was also be the main energy source in seminal plasma (Stefanov 2015). Fructose might also protect sperm from cold shock both inside and outside the cell. This was caused by sugar with big molecule size might serve as intracellular or extracellular cryoprotectant, so that fructose might enter intracellular cryoprotectant and the fructose might penetrate into cell (Klinc & Rath 2006; Paulenz et al. 2009).

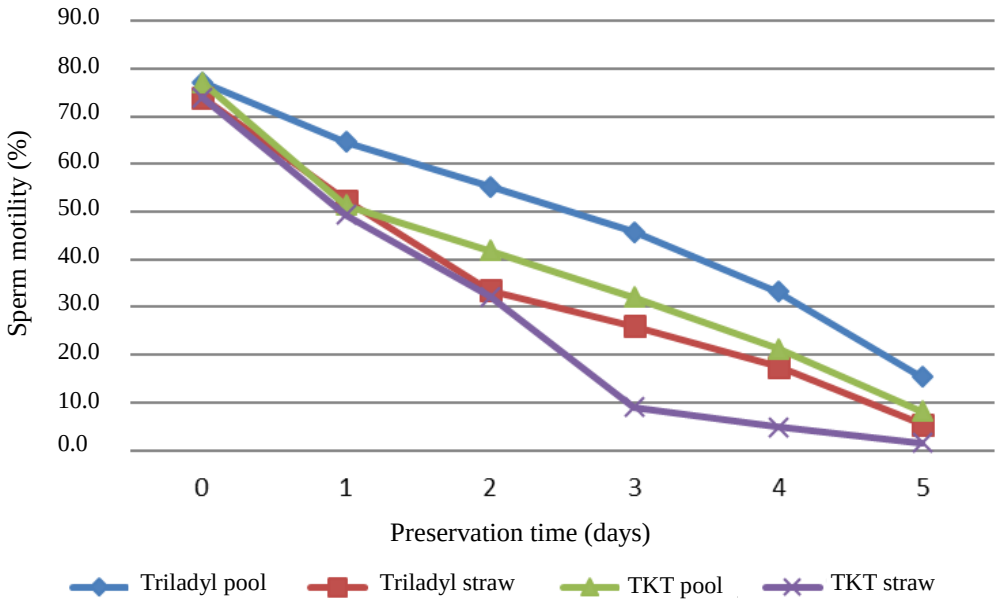

Figure 1. Motility rate of boer goat spermatozoa by different extender and packaging system and preserved at 5°C for 5 days.

Integrity of spermatozoa cell membrane

Generally, integrity of spermatozoa cell membrane of Boer goat preserved at 5°C showed high presentation using Triladyl with both pool or straw packaging method. Difference of integrity rate of membrane in the 4 groups started on the day-2 of preservation. Integrity of spermatozoa membrane using TEY extender with both pool and straw packaging method significantly (P<0.05) decreased up to day-5 of preservation. Integrity membrane rate using Triladyl extender was still good up to day-5 of preservation both with pool (65.5%) or straw (75.0%) packaging (Figure 2). Different result (P<0.05) was showed by the group using TEY both with pool (55.1%) or straw (49.2%) packaging method which showed lower integrity rate of spermatozoa membrane and was not feasible for artificial insemination. Recommended integrity of spermatozoa membrane feasible for the artificial insemination was ≥60% (Revel & Mrode 1994).

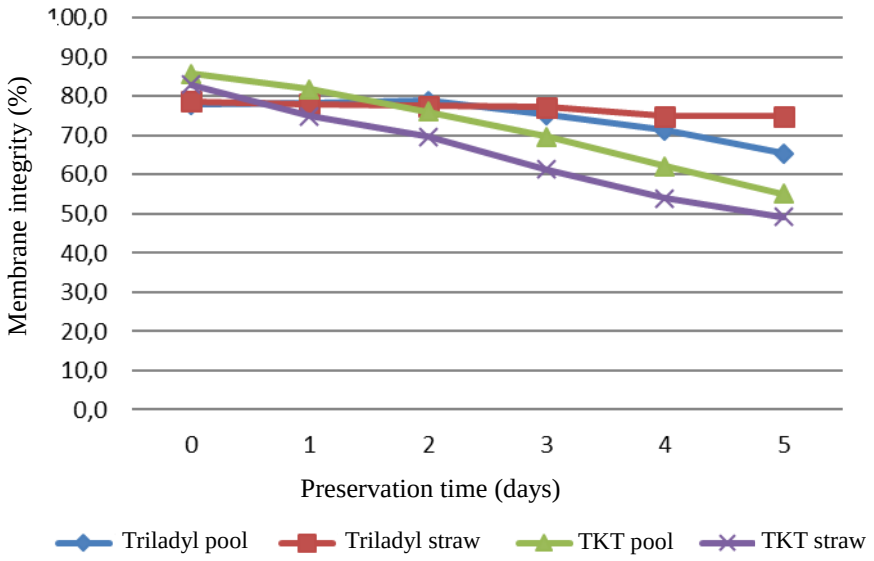

Figure 2. Integrity rate of spermatozoa membrane of Boer goat with different extender and packaging system preserved at 5°C for 5 days.

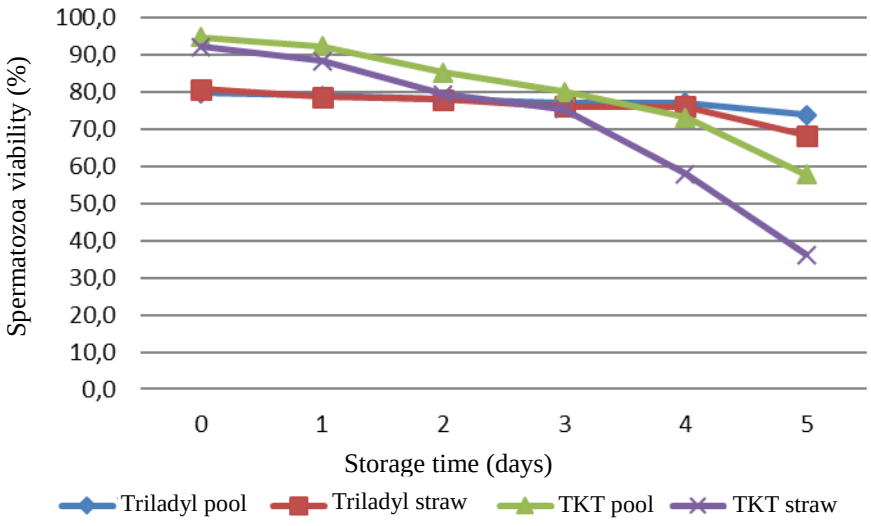

Figure 3. Viability rate of Boer goat with different extender and packaging system preserved at 5°C for 5 days.

Figure 2 shows that percentage of integrity of spermatozoa membrane added by Triladyl both with pool and straw packaging method is higher than other group. It was known that spermatozoa viability was related to integrity of spermatozoa membrane. It was possible that Triladyl extender able to maintain plasma membrane requirement during preservation. It was due to plasma membrane played a role in managing in and out traffic of all substrate and electrolyte needed in metabolic process (Ariantie 2014). Damage in plasma membrane might affect motility and causing death in semen due to aspartate aminotransferase enzyme (AspAT) which was the primary enzyme in mitochondria producing ATP would be released from cell into seminal plasma which would interfere ATP production (Arifiantini & Purwantara 2010).

Sperm viability

Percentage of sperm viability using Triladyl extender started to significantly ($P<0.05$) decrease in day-3 of preservation both with pool (77.1%) and straw (76.2%) packaging method (Figure 3). Therefore, viability of spermatozoa using TEY extender both with pool or straw packaging decreased after day-4 of preservation. Figure 3 shows that decrease of sperm viability percentage using TKT both with pool and straw packaging is significantly ($P<0.05$) lower than sperm viability percentage using Triladyl in day-3 of preservation (73.2% and 58.0%, respectively). The lowest ($P<0.05$) viability was showed by spermatozoa preserved by TEY extender with straw method after day-5 of preservation (36.1%).

Figure 3 shows that Triladyl was able to maintain spermatozoa better than TEY extender. Yolk concentration was lower in Triladyl extender than the Tris. It was assumed that Tris played a role in maintaining spermatozoa viability. Holt et al. (1996) reported that higher concentration of yolk would give negative effect to motility and viability. Especially in goat, phospholipase A enzyme in plasma semen secreted by bulbo-urethralys gland would easily damage semen extender medium especially the one containing of yolk. It caused death of spermatozoa. High concentration of lysolecithin from hydrolysis of yolk lecithin through phospholipase A enzyme snapping in spermatozoa environment caused toxic effect in goat spermatozoa. Leboeuf et al. (2000) said that bulbouretralis gland (Cowper gland) of buck synthesized an enzyme and secreted in plasma semen where coagulation might be occurred if it interacted with yolk. This enzyme was identified as phospholipase A which might hydrolyze lecithin of yolk into fatty acid and lysolecithin poisoning for spermatozoa. Change of spermatozoa viability during preservation for a long term was also strongly related to condition of mitochondria membrane of spermatozoa cell (Love et al. 2003). Mitochondria membrane would damage if there was a composition change in phospholipid and if there was a temperature fluctuation during incubation (Kasimanickam et al. 2012).

The result of this study showed that generally, Triladyl extender was able to maintain chilled semen longer than TEY extender. This was assumed due to the commercial Triladyl extender had better and more complete composition than TEY extender. The result also showed that pool packaging method was better than the straw. It might be caused by difference of ratio of amount extender and concentration or amount spermatozoa in the straw and pool. Concentration of spermatozoa in extender influenced the competition of spermatozoa in nutrient consumption. Metabolism persisted during preservation and causing accumulation of lactic acid increasing pH of medium and causing damage and degrading quality of spermatozoa along with longer time for preservation (Munsi et al. 2007).

CONCLUSION

Triladyl extender with pool packaging was the best method to maintain chilled semen quality of Boer goat which could persist up to day-3 of preservation at 5°C.

ACKNOWLEDGEMENT

Author thanked to Fitra Aji Pamungkas, Rian Rosartio, and technicians who helped during this study.

REFERENCES

Ariantie OS, Yusuf TL, Sajuthi D, Arifiantiny RI. 2014. Kualitas semen cair kambing Peranakan Etawah dalam modifikasi pengencer tris dengan trehalosa dan rafinosa. J Vet. 15:11-22.

Arifiantini RI, Purwantara B. 2010. Motility and viability of Fresian Holstein spermatozoa in three different extender preserved at 5°C. J Indones Trop Anim Agric. 35:222-226.

Alves HM, Oliveira IR, Castelo TS, Lima GL, Souza AL, Moreira AL, de Paula VV, Silva AR. 2013. Comparison of different glycerol and egg yolk concentrations added to tris-based extender for the collared peccaries (*Tayassu tajacu*) semen freezing. Reprod Domest Anim. 48:506-511.

Botham KM, Mayes PA. 2009. Pengangkutan dan penyimpanan lipid. In: Murray RK, Granner DK, Rodwell VW, editors. Harper's Illustrated Biochemistry. 27th ed. Pendit BU, penerjemah. Jakarta (Indones): EGC. p. 225-238.

Holt WV, Abaigar T, Jabbour HN. 1996. Oestrus synchronization, semen preservation and artificial

insemination in the Mohor Gazelle (*Gazella dama mhorr*) for the establishment of a genome resource bank programme. Reprod Fertil Dev. 8:1215-1222.

Ismaya. 2014. Bioteknologi inseminasi buatan pada sapi dan kerbau. Yogyakarta (Indones): Gadjah Mada University Press.

Klinc P, Rath. 2006. Aplication of flowcytometrically sexed spermatozoa in different farm animal species: a review. Archiv Tierzuch Dummerstorf. 49:41-54

Kasimanicham VR, Kasimanicham RK, Memon MA. 2012. Effect of extenders on sperm mitcondrial membrane, plasma membrane and sperm kinetics during liquid preservation of canine semen at 5°C. J Amin Reprod Sci. 136:139-145.

Leboeuf B, Restall B, Salamon S. 2000. Production and storage of goat semen for artificial insemination. Anim Reprod Sci. 62:113-141.

Leethongdee S. 2010. Development of trans-cervical artificial Insemination in sheep with special reference to anatomy of cervix. Suranaree J Sci Technol. 17:157-169.

Love CC, Thompson JA, Brinsko SP, Rigby SL, Blanchard TL, Lowry VK, Varner DD. 2003. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology. 60:1127-1138.

Mara L, Dattena M, Pilichi S, Sanna D, Branca A, Cappai P. 2007. Effect of different extender on sperm motility: collection and goat semen fertility, evaluation of semen. Anim Reprod Sci. 102:152-157.

Medrano A, Garcia-Gill N, Ramio L, Montserrat RM, Pinart E, Concha II, Bonet S, Rigau T, Rodriguez-Gill JE. 2006. Hexose-specificity of hexokinase and ADP-dependence of pyruvate kinase play important roles in the controlof monosaccaride utilization in freshly diluted boar spermatozoa. Mol Reprod Dev. 73:1179-1194.

Munsi MN, Buiyan MMU, Alam MGS. 2007. Effects of exogenous gluthathione on the quality of chilled bull semen. Reprod Domest Anim. 42:358-362

Paulenz H, Soderquist L, Perez R, Berg KA. 2009. Effect of different extenders and sorage temperature on sperm viability of liquid ram semen. Theriogenology. 57:823-836.

Revell SG, Mrode RA. 1994. An osmotic resistance test for bovine semen. Anim Reprod Sci. 36:77-86.

Salomon S, Maxwell WMC. 2000. Preservation of ram semen. Anim Reprod Sci. 62:77-111.

Stefanov RG, Anev G, Abadjieva DV. 2015. Effect of different extenders and strage periods on motility and fertility of ram sperm. Mac Vet Rev. 38:85-89.

Schorin, Marilyn D, Sollid, Kris RD, Edge, Marianne MS, Bouchoux, Ann MSW. 2012. The science of sugar part 1: A closer look at sugars. Nurt Today. 3:96-101.

Taylor MJ. 2006. Biology of cell survival in the cold: The basis for biopreservation of tissues and organs. CRC-Taylor & Francis. p. 15-62.

Verberckmoes S, Van Soom A, Dewulf J, De Pauw I, de Kruif A. 2004. Preservation of fresh bovine semen in extender based on the ionic composition of cauda epydidimal plasma. Reprod Domest Anim. 39:1-7.

Determination of Production Capacity of Circulated Primordial Germ Cells (Circulated-PGCs) of KUB Chicken using Lysis Buffer Ammonium Chloride Potassium (ACK)

Sopiyana S[1], Supriatna I[2], Setiadi MA[2], Fahrudin M[3]

[1]*Indonesian Research Institute for Animal Production, Bogor PO BOX 221, Bogor 16002*
[2] *Department of Reproduction and Pathology Clinic, Faculty of Veterinary Sciences*
Bogor Agricultural University, Jl. Agatis Kampus IPB Darmaga, Bogor 16680
[3] *Department of Anatomy, Physiology, and Farmacology, Faculty of Veterinary Sciences*
Bogor Agricultural University, Jl. Agatis Kampus IPB Darmaga, Bogor 16680
E-mail: soni_sopiyana@yahoo.com

(received 03-02-2016; revised 27-02-2016; accepted 09-03-2016)

ABSTRAK

Sopiyana S, Supriatna I, Setiadi MA, Fahrudin M. 2016. Penentuan kapasitas produksi *primordial germ cell* sirkulasi (PGC-sirkulasi) pada ayam KUB menggunakan metode buffer lisis *ammonium chloride potassium* (ACK). JITV 21(1): 55-61. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1315

Pada embrio unggas, *primordial germ cell* (PGC) merupakan sel progenitor untuk gamet yang memiliki jalur migrasi yang unik. *Primordial germ cell* pertama kali muncul dari epiblast di daerah germinal crescent, kemudian bersirkulasi melalui pembuluh darah dalam periode waktu yang singkat, dan bermigrasi menuju gonad. Penelitian ini bertujuan menentukan kapasitas potensi produksi PGC-sirkulasi embrio ayam KUB pada stadia perkembangan embrio yang berbeda untuk mengisolasi dan mengoleksi PGC-sirkulasi menggunakan metode yang cepat dan sederhana. Dalam penelitian ini digunakan 75 butir telur fertil ayam KUB yang dibagi menjadi lima kelompok perlakuan, dan diinkubasi pada suhu 38,5 ^{0}C dengan kelembaban 60%. Pemanennan embrio diatur sesuai dengan tahapan perkembangan embrio 14-18. Pengambilan darah embrio dilakukan melalui aorta dorsalis dengan menggunakan mikropipet di bawah mikroskop. Darah yang terkumpul ditempatkan pada tabung *eppendorf* 1,5 ml yang telah diisi dengan 100 µl larutan *phosphate buffered saline* tanpa Ca^{2+} dan Mg^{2+} (PBS-) yang dicampur dengan *fetal bovine serum* (FBS) dengan perbandingan 90%:10%. PGC dimurnikan dengan menggunakan proses buffer lisis *ammonium chloride potassium* (ACK). Hasil penelitian menunjukkan bahwa rataan produksi PGC-sirkulasi per embrio dipengaruhi oleh stadia perkembangan embrio (P<0,05). Rataan produksi PGC-sirkulasi dari stadia perkembangan embrio dari 14, 15, 16, 17, dan 18 pada ayam KUB berturut-turut adalah 37,9; 53,5; 49,8; 38,3; dan 33,5 sel per embrio. Produksi PGC-sirkulasi antara stadia 14, 17, dan 18 tidak berbeda nyata (P>0,05). Produksi PGC-sirkulasi terbanyak didapatkan pada stadia 15, sehingga isolasi dan koleksi PGC melalui sirkulasi darah disarankan pada stadia 15.

Kata Kunci: Ayam KUB, PGC, Stadia Perkembangan Embrio, *Ammonium Chloride Potassium*

ABSTRACT

Sopiyana S, Supriatna I, Setiadi MA, Fahrudin M. 2016. Determination of production capacity of circulated primordial germ cells (circulated-PGCs) of KUB chicken using lysis buffer ammonium chloride potassium (ACK). JITV 21(1): 55-61. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1315

In poultry embryos, primordial germ cells (PGCs) are progenitor cells for gametes, which have unique migration pathway. Primordial germ cells arise from epiblast in germinal crescent and circulate through the bloodstream for a short period of time, then leave blood vessel to migrate toward gonads. The aim of this study was to determine the potential production capacity of circulated-PGCs of KUB chicken at different developmental stages of embryo using a rapid and simple method. Seventy five KUB chicken fertile eggs were divided into five groups and incubated at 38.5 ^{0}C with a humidity of 60%. Hatching was set to the embryonic development stage of 14-18. The blood was collected through dorsal aorta using micropipette under microscope. The collected blood was placed in a 1.5 ml eppendorf tube which was previously filled with 100 µl phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS-) mixed with fetal bovine serum (FBS) with a ratio of 90%:10%. The PGCs were purified using lysis buffer ammonium chloride potassium method. The results showed that average production of circulated-PGCs per embryo of KUB chicken were significantly affected by stage of embryonic development (P <0.05). The average production of circulated-PGCs at stage 14, 15, 16, 17, and 18 were 37.9; 53.5; 49.8; 38.3; and 33.5 respectively. The number of circulated-PGCs was not different among stages 14, 17 nor 18. The highest number of circulated-PGCs of KUB chicken was obtained at stage 15, so that the isolation and collection of PGCs through the blood circulation was recommended in stage 15.

Key Words: KUB Chicken, PGCs, Embryonic Development Stage, Ammonium Chloride Potassium

INTRODUCTION

Indonesia is known as one of world megabiodiversities. Indonesian native poultry is one of animal genetic resources which potential for egg and meat, and fancy animal. Sartika & Iskandar (2007) reported that there were 43 recognized native chicken breeds in Indonesia, which can be further developed and preserved in-situ and ex-situ. Since 2012 Indonesian Research Institute for Animal Production has developed a strain derived from native Kampung chicken breed as improved egg producers strain. KUB chicken is the name of the strain which was able to produce 44.33% henday production, reaching peak production of 60% (Iskandar & Sartika 2014). As a new strain, KUB chicken may be categorized as new genetic resources that is needed to be conservated.

Conservation activity was generally done through in-situ method where the maintenance was conducted in its natural habitat and ex-situ method by maintaining live animals outside its natural habitat. Somehow, in-situ and ex-situ conservations of live animals require high maintainance cost and disease risks.

Conservation technique on semen, ovum even embryo in bird did not come to satisfy than when applied in mammal. Blesbois & Labbe (2003) reported that percentage of live sperm after thawing was only 60%. Ovum freezing in bird still was not succeeded regarding its big size and complexity and high fat content in bird (Blesbois & Labbe 2003). Bird's ovum had some issues causing cryopreservation might not be done (Hagedorn et al. 2004). An approach currently developed to solve limitation on in-situ and ex-situ conservations especially in bird is primordial germ cells (PGCs) cryopreservation. Cryopreserved primordial germ cells might be preserved for unlimited time and might be used everytime needed.

Primordial germ cells were gamet progenitor cells for or spermatozoa and ovum progenitor and were early development of gamet cells (Qian et al. 2010; Glover & McGrew 2012; Tajima 2012). Qian et al. (2010) reported that PGCs was formed out of gonad and migrated toward gonad during embryogenesis which will be developed into spermatozoa and ovum. Primordial germ cells might be used as genetic source and for establishing transgenic chicken (Furuta 2012). Primordial germ cells were cells with plurypotent characteristic making the PGCs as a good model to study embryonic development by in-vitro (Wang et al. 2010).

Primordial germ cells were first detected in chicken embryo in the end of gastrulation (Ginsburg 1997) and first appeared from epiblast and first located in ventral part of pelucyde area of embryo stage X, then it was translocated into dorsal side of hypoblast in stage XI-XIV or stage 2 to 6 (Hamburger & Hamilton 1951), then was migrated into germinal crescent area in stage 4 through bottom layer. PGCs migration was stoped at the end of gastrulation (Ginsburg 1997). During blood vessel forming, PGCs entered blood vessel between stage 10 and 12 and begun to circulate in bloodstream. During stage 12 to 16, PGCs was stay still in bloodstream and migrated into gonad in stage 20-24, where they begin to differentiate into male and female gametes (Chojbacka-Puchta et al. 2012).

Chojbacka-Puchta et al. (2012) reported that PGCs might be divided into 3 types based on time and place of isolation, there were (1) blastoderm or stage X; (2) circulation of embryo blood after eggs incubated for 2.5 to 3 days (stage 13-16 HH) known as circulated-PGCs); (3) gonad embryo after eggs incubated for 5.5 to 6 days (stage 26-28 HH) known as gonad-PGCs.

Unique migration path of chicken PGCs easy PGCs collection based on its place. A method to collect PGCs from chicken widely used was by isolating PGCs from 2.5-3 days old embryo blood (Yamamoto et al. 2007; Nakamura et al. 2007). The number of circulated-PGCs would be different in every embryonic development stage (Tajima et al. 1999; Li et al. 2001). Zhao & Kuwana (2003) reported that the number of circulated-PGCs in embryonic development stage would be affected by variated egg production.

The most embryonic development stage with circulated-PGCs of chicken was in stage 13-18 (Tajima et al. 1999), stage 13 in quail (Li et al. 2001), stage 15 in White Leghorn chicken (Kuwana et al. 2006), and stage 14 in Kureko Dori chicken (Qian et al. 2010). Information of embryonic development stage and circulated-PGCs in native chicken was varied among 15-29 cells. The most number of circulated-PGCs was found in Indonesian native Gaok chicken in stage 15 by 51 cells per embryo (Kostaman 2013a).

This study was to determine embryonic development stage with the highest circulated-PGCs production of KUB chicken using buffer lysis ACK. This study result was expected to be basic of proper embryonic development stage determination in native chicken in isolating PGCs through blood vessel using buffer lysis ammonium chloride potassium (ACK) method.

MATERIALS AND METHODS

Time and place

This study was conducted on April to August 2015 in Laboratory of Genetic and Livestock Animal Germplasm, Indonesian Research Institute for Animal Production (IRIAP), Ciawi-Bogor.

Fertile eggs preparation

Seventy five fertile eggs of KUB chicken from Chicken Research laboratory of IRIAP, Ciawi-Bogor were used in this study for circulated-PGCs collection. Those eggs were divided into 5 treatment groups based on embryonic development stage at 14-18 hours (there were 5 embryonic development stages by 5 eggs in each group). Eggs were incubated in 38.5°C with 60% humidity and stirred as 90°C every 30 minutes using portable incubator (P-008B Biotype; Showa Furanki, Saitama, Jepang). Embryo collection from each treatment was set as embryonic development stage, that was in 14-18 HH (Hamburger & Hamilton 1951) or after 50-72 hours incubation.

Isolation and collection of circulated-PGCs using buffer lysis ACK

After egg reached desired embryonic development stage, eggshell was broken and the content was moved into plastic petri dish (90 x 15 mm, LBS60001PT, BIOLAB). Embryo blood was collected through dorsalis aorta using micropipette (50 µm; Drummond Scientific, Broomall, PA USA) under microscop (Olympus SZX7, Japan). Dorsalis aorta was near the heart and providing blood in a large amount. The accumulated blood was divided based on embryonic development stage (14-18 HH) and each was put in *eppendorf* 1.5 ml tube filled by 100 µl phosphate buffered saline solution without Ca^{2+} and Mg^{2+} (PBS-) + fetal bovine serum (FBS) with ratio by 90%:10%. PGCs was purified using buffer lysis ACK process (Yamamoto et al. 2007) which was new method to be developed to isolate circulated-PGCs from embryo blood circulation. This was an easy and fast method to purify circulated-PGCs using ammonium chloride-potassium (ACK).

Variables evaluated in this study were morphology characteristic and production of circulated-PGCs of KUB chicken determined by standard reported by Hamburger & Hamilton (1951) and based on incubation time, embryonic morphology form, the number of somit, and embryonic development. Circulated primordial germ cells might be distinguished from red blood cells apart from cell size and other characteristics such as nucleus, refractive fat, or ring of surrounding cells.

Evaluation and analysis of study results

Circulated-PGCs isolation result as morphology characteristic was descriptively analyzed. Production potential of circulated-PGCs from each embryonic development stage (stage 14, 15, 16, 17, and 18) was statistically analyzed using Completely Randomized Design with 15 replications. Data were analyzed by ANOVA which its normality was analyzed by Kolmogorof-Smirnov Z before. If there was different between those treatments, it was continued by Duncan multiple range test (Steel & Torrie 1995).

RESULT AND DISCUSSION

Issolation and identification of circulated primordial germ cells (circulated-PGCs) using buffer lysis ACK

Circulated PGCs were isolated from embryo blood after egg incubated for 2.5-3 days (stage 14-18, Hamburger & Hamilton 1951). In this study, circulated-PGCs of KUB chicken has been successfully isolated and identified using buffer lysis ACK from each blood sample in stage 14-18.

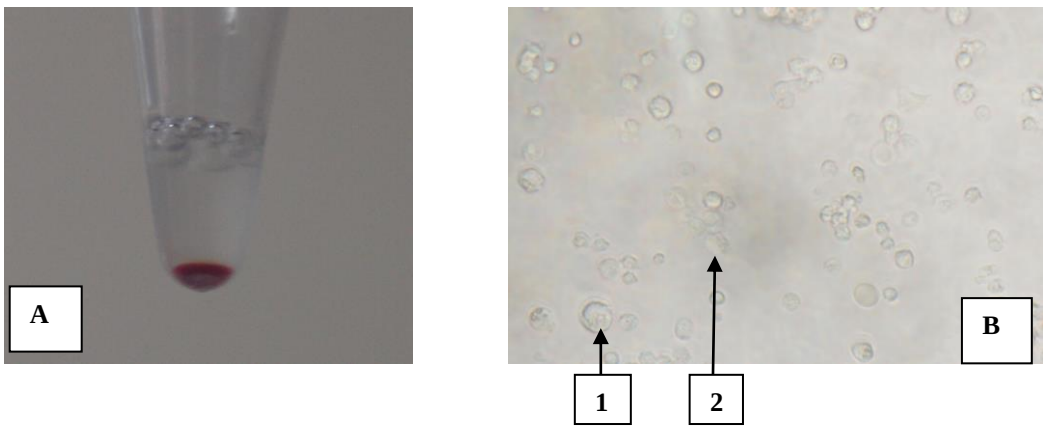

Figure 1. Issolation of circulated-PGCs of KUB chicken using buffer lysis ACK method. A) Blood sample collected from 14-18 stadium of embryonic development. B) First purification of ACK process and centrufigation, circulated-PGCs which is still mixed with red blood cells. 1=circulated-PGCs and 2=red blood cells.

Table 1. Comparation of morphology characteristic between circulated-PGCs and red blood cells

Chriteria	Circulated-PGCs	Red blood cells (erythrocytes)
Size (µm)[1)]	14-19	6-8
Cell form	Oval or round, irregular contours	Round
Core	Spherical, large, asymmetrical, clear	Exist, not clear
Specific gravity[2)]	1.029-1.040	1.054-1.060
Fat *Droplets*	There were fat *droplets* in cytoplasm	Not exist
Ring	Teher was light ring surrounds the cell	Not exist

Source: [1)]Yashuda et al. (1992); [2)]Qian et al. (2010)

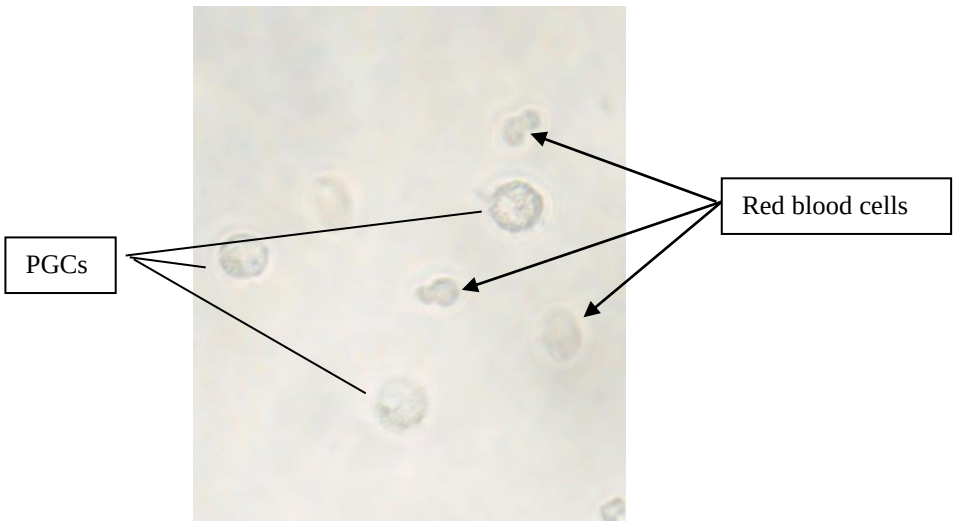

Figure 2. Circulated-PGCs of KUB chicken obtained using buffer lysis ACK (magnification by 400x).

The result from descriptive analysis showed that circulated-PGCs had the same morphological characteristic with the one reported by Ginsburg (1997); Yashuda et al. (1992); Kostaman (2013b) in previous study which showed that PGCs in chicken might be identified morphologically as (1) it had bug cells (14-19 µm), (2) oval or round and irregular contours, (3) there was light fat droplets distributed in sitoplasm, (4) sperical and big core located unsymmetrically and containing prominent nucleous, (5) there was light ring under cell membrane on the outskirts of PGCs.

Other information reported that generally, basic characteristic of PGCs in chicken was about the same with in quail, pheasant, and duck. By those characteristics, PGCs might be distinguished with red blood cells as in Table 1.

In this study, circulated-PGCs was purified using buffer solution and centrifugation with speed developed by Yamamoto et al. (2007) consisting of two time purifications and two time flushings. Main principle of this purification method was dividing red blood cells with circulated-PGCs by lysising red blood cells where ACK process would dissolve red blood cells and did not affect circulated-PGCs morphology.

Buffer lysis ACK solution consisted of *ammonium chloride*, *potassium bicarbonate*, and EDTA. Ammonium choride was anorganic compound with as NH_4Cl as white crystal salt which was highly water soluble. Potassium bicarbonate was a colorless, odorless and salty substance. EDTA was an anticoalgulant of an odorless aminopolycarboxylic acid and a solid substance dissolved in water. Buffer lysis ACK solution was an odorless substance used to lysis red blood cells and did not affect the circulated-PGCs morphology (Yamamoto et al. 2007).

Table 2. Production of circulated-PGCs per embryo using buffer lysis ACK method in KUB chicken

Embryonic development stage (HH)	Incubation time (hour)	Average of circulated-PGCs per embryo (cells)
14	50-52	37.9±9.2[a]
15	53-55	53.5±8.0[b]
16	56-59	49.8±6.2[b]
17	60-63	38.3±4.3[a]
18	64-67	33.5±5.7[a]

Numerals in the same column followed by the same letter show not significantly different at 5% (Duncan Multiple Range Test)

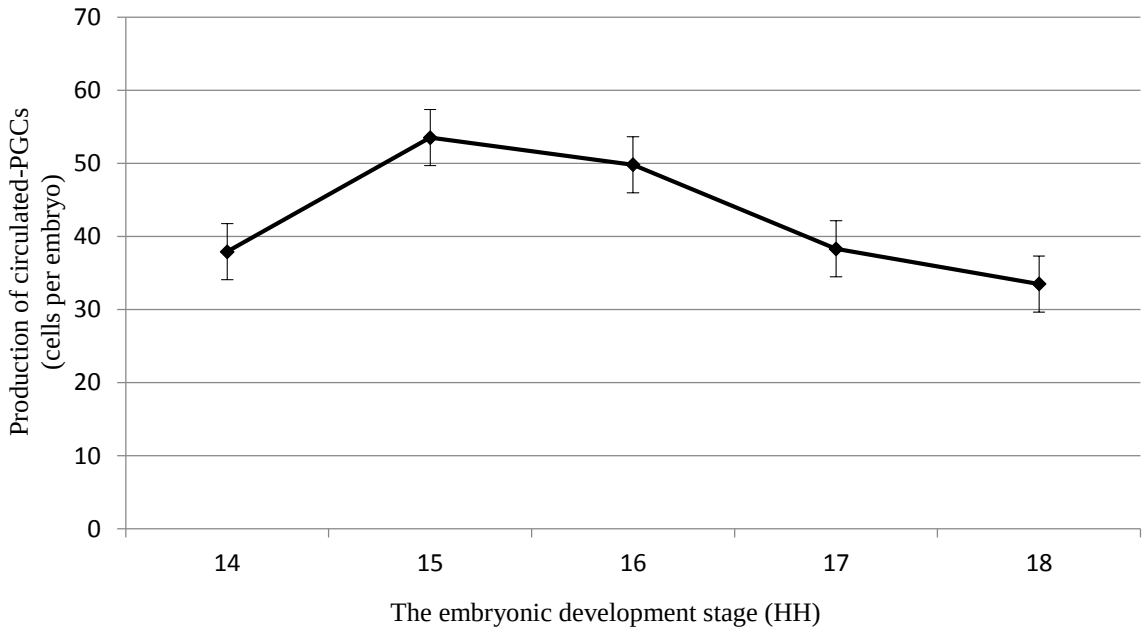

Figure 3. Circulated-PGCs of KUB chicken in different incubation time.

Circulated primordial germ cells (Circulated-PGCs) of KUB Chicken

Based on isolation obtained and calculation of circulated-PGCs per embryo, it was showed that the number of circulated-PGCs varied between embryonic development stage (Table 2).

The highest average number of circulated-PGCs ($P<0.05$) was found in stage 15 and 16 compared to stage 14, 17, and 18 where there was varied average circulated-PGCs amount in each embryonic development stage. This showed that circulated-PGCs production was affected by embryonic development stage.

From stage 14, production of circulated-PGCs in embryo blood sample was increase and reached the peak at stage 15. At stage 15, it was possible that all PGCs gathered in embryo circulatory system (Kostaman et al. 2013a). The peak of PGCs production persisted until stage 16 and decrased afterword. This was proved by statistic analysis result which showed that the number of PGCs production at stage 15 and 16 did not show significant different ($P>0.05$). Circulated-PGCs continued to decrease at stage 17 and a little reminded in embryo blood circulatory system. This was in accordance with a research conducted by Kostaman et al. (2013a) in Indonesian native Gaok chicken showing that the number of circulated-PGCs would decrease after stage 16-18. This was possible to be caused by PGCs characteristic which had unique migration path circulating in a short time in blood circulation system and immediately left blood vessel into gonad (Yamamoto et al. 2007). This was also in accordance with the result in this study which showed that circulated-PGCs reached the peak in blood circulation system at stage 15 and would decrease after that. It was possible that most PGCs have begun left the blood circulation system. Decrease of the number of

circulated-PGCs in embryonic development stage 14-18 HH (Figure 3).

This result was not significantly different with previous study in Indonesian native Gaok chicken reporting that the number of circulated-PGCs, when purified by nicodenz density gradient sentrifugation method reached peak amount at stage 15 to of 51.0 cells per embryo (Kostaman 2013a). That result was a considerably lower than previous research result reported that the most circulated-PGCs in Rhode Island Red (RIR) chicken blood circulation system was 67-73 cell at the stage of 15 (Nakamura et al. 2007), and it was different from Silky chicken where the circulated-PGCs peak amount was at stage 14 by 65 cells (Qian et al. 2010). Setioko (2007) reported that average amount of PGCs in White Leghorn chicken was 84.2 cells. Some research results showed that the number of PGCs of native chicken was varied between 15-51 cells (Setioko et al. 2010; Kostaman 2013a). It also showed that appropriate time to harvest the circulated-PGCs was in stage 15 (53.5 cells). At that stage, circulated-PGCs might be obtained in the largest number. This was an appropriate time to preservate PGCs and form germline chimera.

In this study, embryo blood collection did not begin at stage 13, but at stage 14 where the amount of circulated-PGCs at stage 13 was lower due to the extremely smooth blood vessel of embryo (Li et al. 2001). Collection of embryo blood was terminated on stage 19 because the PGCs still remained in the blood vessels. The stage 20 of the most PGCs had left the blood vessels and had begun to arrive in the gonadal anlage (Ukeshima et al. 1991; Ginsburg 1997). Collection of embryo blood at the stage 19 would be difficult due to the blood vessel already thickened, so that it would be difficult to inject and would be broken when imposed.

CONCLUSION

The highest number of circulated-PGCs in KUB chicken was in stage 15 and this stage was the best time to isolate PGCs to obtain maximum circulated PGCs.

ACKNOWLEDGEMENT

Authors thanked to Dr. Tatan Kostaman, Dr. Tike Sartika, Prof. (R) Dr. Sofjan Iskandar, and Dr. L. Hardi Prasetyo who helped both material and non-material until this research and article finished.

REFERENCES

Blesbois E, Labbé C. 2003. Main improvements in semen and embryo cryopreservation for fish and fowl. In: Planchenault D, editor. Cryopreservation of Animal Genetic Resources in Europe, Paris, France. p. 55-65.

Chojbacka-Puchta L, Kasperczyk K, Pluciennicza G, Sawicka D, Bednarczyk. 2012. Primordial germ cells (PGCs) as a tool for creating transgenic chickens. Polish J Vet Sci. 15:181-188.

Furuta H. 2012. Establishing germline chimeric chickens using primordial germ cells. J Poult Sci. 49:1-4.

Ginsburg M. 1997. Primordial germ cells development in avians. J Poult Sci. 76:91-95.

Glover JD, McGrew MJ. 2012. Primordial germ cells technologies for avian germplasm cryopreservation and investigating germ cell development. J Poult Sci. 49:155-162.

Hagedorn M, Peterson A, Mazur P, and Kleinhans FW. 2004. High ice nucleation temperature of zebrafish embryos: slow-freezing is not an option. Cryobiology. 49:181-189.

Hamburger V, Hamilton HL. 1951. A series of normal stages in development of the chick embryo. J Morphol. 88:49-92.

Iskandar S, Sartika S. 2014. KUB Chicken: The first Indonesia Kampung chicken selected for egg production. Proceedings of the 16th AAAP Animal Science Congress Vol II 10-14 November 2014. Yogyakarta (Indones): Gadjah Mada University. p. 157-160.

Kostaman T, Yusuf TL, Fahrudin M, Setiadi MA. 2013a. Isolasi dan jumlah *primordial germ cell* sirkulasi (PGC-sirkulasi) pada stadium perkembangan embrio ayam Gaok. JITV. 18:27-33.

Kostaman T. 2013b. Isolasi dan kriopreservasi *primordial germ cell* (PGC) menggunakan kriopotektan DMSO untuk pembentukan germline chimera ayam Gaok (Dissertation). [Bogor (Indones)]: Institut Pertanian Bogor.

Kuwana T, Kawashima T, Naito M, Yamashita H, matsuzaki M, Takano T. 2006. Conservation of a threathened indigenous fowl (Kureko Dori) using the germline chimeras transpanted from primordial germ cells. J Poult Sci. 43:60-66.

Li HC, Matsui K, Ono T. 2001. Population of circulating primordial germ cells in early Japanese quail embryos. J Poult Sci. 38:175-180.

Nakamura Y, Yamamoto Y, Usui F, Mushita T, Ono T, Setioko AR, Takeda K, Nirasawa K, Kagami H, Tagami T. 2007. Migration and proliferation of primordial germ cells in the early chicken embryo. J Poult Sci. 86:2182-2193.

Qian C, Zhau Z, Han H, Zhao C, Jin X, Zhao H, Zhang Y, Chen W, Yang N, Li Z. 2010. Influence of microgravity on the concentration of circulating primordial germ cells in Silky chicken offspring. J Poult Sci. 47:65-70.

Sartika T, Iskandar S. 2007. Mengenal plasma nutfah ayam Indonesia dan pemanfaatannya. Edisi Pertama. Bogor (Indones): Balai Penelitian Ternak.

Setioko AR, Tagami T, Tase H, Nakamura Y, Takeda K, Nirasawa K. 2007. Cryopreservation of primordial germ cells (PGCs) from White Leghorn embryo using commercial cryoprotectants. J Poult Sci. 44:73-77.

Setioko AR, Kostaman T, Sopiyana S. 2010. Jumlah *primordial germ cell* (PGC) pada beberapa tingkat umur embrio yang berbeda pada ayam buras dan ras. Prosiding Seminar Nasional Biologi. Bandung (Indones): Universitas Padjajaran. hlm. 133-141.

Steel RGD, Torrie JH. 1995. Prinsip dan prosedur statistika: Suatu pendekatan biometrik. Sumantri B, penerjemah. Principles and procedurs of statistics: A biometrical approach. Jakarta (Indones): Gramedia Pustaka Utama.

Tajima A, Hayasi H, Kamizumi A, Ogura J, Kuwana T, Chikamune T. 1999. Study on the concentration of circulating primordial germ cells (cPGCs) in early chick embryos. J Exp Zool. 28:759-764.

Tajima A. 2012. Conservation of avian genetic resources. J Poult Sci. 50:1-8.

Ukeshima A, Yoshinaga K, Fujimoto T. 1991. Scanning and transmission electron microscopic observations of chick primordial germ cells with special reference to the extravasation in their migration course. J Elec Micros. 40:124-128.

Wang Y, Hou L, Li C, Guan W, Chen L, Li X, Yue W, Ma YH. 2010. Isolation, culture, and biological characteristics of primordial germ cells from Beijing Fatty chicken. J Reprod Dev. 56:303-308.

Yamamoto Y, Usui F, Nakamura Y, Ito Y, Tagami T, Nirasawa K, Matsubara Y, Ono T, Kagami H. 2007. A novel method to isolate Primordial Germ Cells and its use for the generation of germline chimeras in chicken. Biol Reprod. 77:115-119

Yashuda Y, Tajima A, Fujimoto T, Kuwana T. 1992. A method to obtain avian germline chimeras using isolated primordial germ cells. J Reprod Fertil. 96:521-528.

Zhao DF, Kuwana T. 2003. Purification of avian circulating primordial germ cells by Nycodenz density gradient centrifugation. Br Poult Sci. 44:30-35.

Potency of Antigenic and Serologic Tests Based on CNTKCQTP Linear Epitope on H5N1 Haemagglutinin for Avian Influenza

Tarigan S, Sumarningsih

Indonesian Research Center for Veterinary Sciences
E-mail: simsont@me.com

(received 18-11-2015; revised 23-02-2016; accepted 03-03-2016)

ABSTRAK

Tarigan S, Sumarningsih. 2016. Potensi tes antigen dan tes serologi yang didasarkan pada epitope linier CNTKCQTP pada haemagglutinin H5N1 untuk Avian Influenza. JITV 21(1): 62-72. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1251

Alat diagnosa cepat atau *point-of-care* (POC) *test* sangat dibutuhkan dalam usaha pengendalian dan pemberantasan *high pathogenic avian influenza* (HPAI) H5N1 di Indonesia. Akurasi alat diagnosa POC ditentukan oleh spesifitas antibodi yang merupakan komponen utama dari semua alat diagnosis cepat. Baru-baru ini dilaporkan bahwa epitop CNTKCQTP yang terletak pada residu asam amino 274-281 protein hemaglutinin H5 hanya dimiliki oleh virus H5N1 dan semua clade virus subtipe H5N1 memiliki epitop tersebut. Penelitian ini bertujuan memproduksi dan mengevaluasi spesifitas poliklonal antibodi terhadap epitope tersebut. Antibodi diproduksi dengan cara mengimunisasi kambing dengan peptida tersebut dalam bentuk multiple antigen peptide (MAP). Spesifitas antibodi diukur dengan cara menganalisis reaktivitasnya terhadap virus influenza subtipe H3N3, H4N4, H5N1, H6N5, H7N7, H9N2, H10N7 dan H11N9, dan rekombinan hemagglutinin H1-H12, H14 dan H15 dengan ELISA dan immunoblot. Hasil ELISA dan immunoblot menunjukkan bahwa antibodi CNTKCQTP tidak spesifik terhadap haemagglitinin H5 karena memiliki reaksi silang dengan haemgglutinin lain terutama H7, H8 dan H9. Potensi peptida yang mengandung epitop, GNCNTKCQTPMGAINSS. sebagai reagen ELISA untuk mengukur antibodi H5 pada ayam yang sebelumnya telah divaksin dan ditantang dengan virus H5N1 juga dievaluasi dalam penelitian ini. Berbeda dengan hasil peneliti sebelumnya, ELISA yang menggunakan peptida tersebut sebagai *coating* atau pelapis tidak sensitif mendeteksi *antibody* haemagglutinin H5 pada ayam.

Kata Kunci: Virus AI, Hemagglutinin H5, Epitop CNTKCQTP, MAP, Immunoassay

ABSTRACT

Tarigan S, Sumarningsih. 2016. Potency of antigenic and serologic tests based on CNTKCQTP linear epitope on H5N1 haemagglutinin for Avian Influenza. JITV 21(1): 62-72. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1251

Rapid diagnostic tools or point-of-care (POC) test is needed in the effort to control and eradicate the high pathogenic avian influenza (HPAI) H5N1 in Indonesia. Accuracy of a POC test is determined by the specificity of antibodies, which is the main component of a POC test. Recently a linear epitope, CNTCKQTP epitope, located at 274-281 amino acid residue of H5 hemagglutinin has been confirmed to be present all clade of H5N1 viruses. This study aimed at producing and evaluating the reactivity of a monospecific, polyclonal antibody against the epitope. The Antibody was produced by immunizing a goat with the peptide in the form of multiple antigen peptide (MAP). The specificity of the antibody was estimated by assaying its reactivity against influenza virus subtypes H3N3, H4N4, H5N1, H6N5, H7N7, H9N2, H10N7 and H11N9; and recombinant hemagglutinins H1-H12, H14 and H15 with ELISA and immunoblot. The results of the assay showed that CNTKCQTP antibody was not specific for H5 haemagglitinin because it cross-reacted with other haemagglutinins especially H7, H8 and H9. The potential of the peptide containing the epitope, GNCNTKCQTPMGAINSS. as an ELISA reagent for assaying H5 antibodies in chickens previously vaccinated and challenged with the H5N1 virus was also evaluated in this study. In contrast the results of previous studies, the ELISA using GNCNTKCQTPMGAINSS as coating antigen was not sensitive in detecting antibody to haemagglutinin H5 in chickens.

Key Words: AI Virus, Hemagglutinin H5, CNTKCQTP Epitope, MAP, Immunoassay

INTRODUCTION

In the early 2000s, highly pathogenic avian influenza (HPAI) H5N1 influenza was detected in many parts of the world causing concerns particularly after eighteen people were hospitalized, six of whom died, after contracting the H5N1 virus from infected chickens (Claas et al. 1998). Between December 2003 and January 2004, outbreaks of H5N1 sprang up simultaneously in eight countries in the East and Southeast Asia (Sims et al. 2005). Within three years, the disease has spread to 63 countries (FAO 2012) and caused death and culling of about 400 million domestic poultry globally with estimated economic losses of about US $20 billion (FAO 2012). Most of the 63 affected countries were able to eliminate H5N1 virus

rapidly except for China, Indonesia, Vietnam, India, Bangladesh and Egypt, where eradication of the disease proven to be difficult and subsequently has become endemic (FAO 2011).

One of the most important factors for those countries that successfully eradicated H5N1 virus was their ability to quickly recognise the disease enabling rapid elimination of all infected birds. To be able to recognize a disease quickly, the availability of appropriate diagnostic techniques is of paramount importance. For countries where the H5N1 has became endemic, two types of diagnostic tests are necessary; the first type that includes virus isolation and real time PCR, is intended for confirmatory, unambiguous diagnosis, whereas the second type is the point-of-care, or pen-site test intended for rapid diagnosis and to guide in determining actions needed to be taken for control or eradication of disease (Tarigan 2015). Most tests of the second type are immunological test based on the use of an antibody to detect the presence of H5N1 antigen.

To obtain an immunological test with high specificity, the use of monoclonal antibody is the preferred choice (Ho et al. 2009; Lin et al. 2015). However, production of suitable monoclonal antibody is expensive and requires high technical skills.

On the other hand however, the specificity, which is the main advantage of monoclonal antibody, may in some circumstances become a disadvantage. A monoclonal antibody may be so specific that it could not tolerate even a minute change (single amino acid change) in its epitope and this reduces its sensitivity when used in a diagnostic test (Khan 2014; Singh et al. 2014). Thus, in some instances a polyclonal antibody is more suited than a monoclonal antibody, especially when the polyclonal antibody has been generated against a small, unique antigen.

Recently, a linear epitope at amino acid residues 274-281 (CNTKCQTP) in the in HA1 region of the haemagglutinin specific for the H5N1 subtype viruses has been identified (Prabakaran et al. 2009). The epitope was shown to be 100% conserved among 163 human isolate and 96.9% in 906 avian isolate of H5N1 viruses. This epitope is not present in H2, H3, H4 and H6 - H16 subtypes of influenza viruses, or that the same sequence is present only in a small number (2.3%) of H1 subtype strains. The CNTKCQTP epitope, in the form of a synthetic peptide, immobilized on a microtitre-plate reacted in ELISA with sera from birds vaccinated with different clades of H5N1 viruses (Velumani et al. 2011). The ELISA was reported to be 100% specific and recommended to be use as a tool in surveillance of H5N1 influenza in human and animals (Velumani et al. 2011).

The fact that the CNTKCQTP epitope is specific for the haemagglutinin of H5N1 viruses leads us to assumption that an antibody raised to that epitope may be useful as a diagnostic reagent in particular for pen-site test intended for rapid H5N1 diagnosis in poultry. In this study we generated an antibody by immunizing a goat with a synthetic peptide CNTKCQTP and analyzed its reactivity against haemagglutinins of different subtypes of avian influenza viruses. The ultimate goal of this study was to evaluate the potency of the goat-anti- CNTKCQTP epitope as the main reagent for immunodiagnostic test for H5N1 avian influenza.

MATERIAL AND METHODS

Peptide

Two forms of CNTKCQTP peptide were used in this study. The first was as a multiple antigenic peptide (MAP), a four-symmetrical-branched, 8-amino-acid (aa)-long (CNTKCQTP) peptide with unknown purity. This aa sequence is found in the haemagglutinin of all H5N1 viruses at aa position 274-281 (Prabakaran et al. 2009; Velumani et al. 2011). The second form was as a single linear 17-aa-long (GNCNTKCQTPMGAINSS) peptide with >95% purity. This aa sequence is found in the haemagglutinin of all H5N1 viruses, at aa position 272-288 (Prabakaran et al. 2009; Velumani et al. 2011). Both peptides were synthetised by VCPBIO Ltd. Shenzhen City, China.

Production and measurement of anti-CNTKCQTP-antibody

A one year old, Ettawah-cross breed, male goat was purchased from a nearby farm. The goat was kept in a stilted, slitter floor isolated from other animal species, fed on natural foliage and grass supplemented with commercial concentrate (0.1 kg/day). After adapting to the laboratory condition for 3 weeks, the goat was immunized subcutaneously with 2 mg CNTKQTP MAP in complete Freund's adjuvant. Booster Immunisations was carried out at 4 and 8 weeks after the first immunization with the same amount of peptide but with incomplete Freund's adjuvant. The fourth immunization was carried out 12 week after the first immunization with 4 mg CNTKQTP MAP and Quil A adjuvant (Superfos Biosector, Denmark) intramuscularly. The goat was bled before each vaccination. Two weeks after the last immunization, the goat was humanely exsanguinated. Serum was aliquoted in 10 ml tubes and stored at -20°C.

The level of anti-CNTKCQTP antibody in the serum of the goat before and after immunizations was measured with an indirect ELISA. The CNTKQTP MAP or GNCNTKCQTPMGAINSS single peptide was first diluted in 0.1 *M* carbonate-bicarbonate buffer (pH 9.6) at 5 µg/ml. Microtitre plates (Nunc Maxisorp^{R})

were coated with the peptide at 100 µl/well at 4°C overnight. After blocking with non-fat skim milk (5 mg/ml) for 2 hours, serially diluted goat serum in PBS containing normal rabbit serum (20 µl/ml) was added and incubated at room temperature (25°C) for 2 hrs. After washing four times with PBST (0.05% Tween-20 in PBS, pH 7.2), rabbit- anti-goat-IgG- HRP conjugate (Sigma Co. Singapore), diluted in PBS-NRS at 1:4000, was added. After washing four times with PBST, solution of ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and H_2O_2 substrate was added and the absorbance (A_{420}) measured with a microtitre-plate reader.

Measurement of antibody level to CNTKCQTP in naïve, vaccinated and challenged chickens

Sera

Sera from naïve, vaccinated and H5N1 infected chickens were obtained from an experiment conducted in our Laboratory and described previous (Tarigan et al. 2015). Briefly, layer chickens were vaccinated with a commercial-killed-H5N1 vaccine (Medivac-AI®, PT Medion, Bandung, Indonesia). Two weeks after the last vaccination, birds were challenged with an isolate of H5N1 virus (A/Chicken/WestJava/Sbg-29/2007). Birds were bled one day before vacciation, two week after the first, second and third vaccination, and every week, from 1 to 8 weeks, after challenge. For the current study, 20 randomly selected sera were used from each, prevaccination (naïve sera), after 1st, 2nd and 3rd vaccination (vacinated sera), one week after H5N1 challenge (early stage infection sera), 2- or 3- weeks after challenge (mid stage of infection sera) and >4 week after infection (late stage of infection sera). In addition, sera from two specific pathogen-free (SPF) chicken were also included in the assays (PT Vaksindo Satwa Nusantara, Indonesia).

Measurement of antibody level to CNTKCQTP

Antibody to CNTKCQTP in the bird's sera was measured by an indirect ELISA. Microtitre plate was coated with CNTKQTP MAP or GNCNTKCQTPMGAINSS single peptide and blocked as described previously for the immunized-goat sera. Bird's sera diluted 1 : 100 were added to the plates and incubated for 2 hours. After washings four times with PBST, rabbit-anti-chicken IgG- HRP- conjugate (Sigma Co. Singapore) diluted at 1 : 5000 in PBST was added, then incubated at 37°C for 2 hours. In addition to the sera from the experimental birds, serum from SPF chickens were also added to the plates, as additional negative control. After washings, solution of substrate and chromogenic ABTS was added and the absorbance (A_{420}) meassured with a microtitre plate reader.

Immunoblotting

Immunoblot assay was used to analyse the reactivity of the CNTKQTP- goat antiserum with the different sub-types avian influenza viruses. The first immunoblot was between the serum and proteins of whole avian influenza viruses. The avian influenza virus subtypes available for this study included: (1) A/Avian/669/WA/78 (H3N8), (2) A/Grey Teal/WA/1840 (H4N4) (3) A/Chicken/WestJava/Sbg-29/2007(H5N1), (4) (A/Shearwater/Australia/1/72 (H6N5), (5) A/Duck/Victoria/76/Keysborough (H7N7), (6) A/Turkey/Wisconsin/66 (H9N2), (7) CSIRO, AAHL GI (H10N7), (8) A/Tern/Aust/75 (H11N9). All viruses had been inactivated as they were prepared and used in haemagglutination inhibition (HI) test. All viruses, except for the H5N1 subtype viruses, were a kind gift from Dr. Peter Durr (Australian Animal Health Laboratory, Geelong, Australia). Before loading to the SDS-PAGE, the inactivated viruses were treated according to a previous method (Rosenberg, 1996b). Briefly, 400 µl methanol was added to 100 µl virus suspension, vortexed briefly and spun at 14000 x *G* for 10 second in a microfuge. After adding 150 µl chloroform, the samples were vortexed and spun at 14000 x *G* for 10 seconds. After adding 300 µl distilled H2O and vortex, the samples were spun at 14000 x *G* for 30 second. The liquid above the protein band at the interface between H_20 and chloroform was discarded, and 300 µl methanol was added. Protein was pelleted at 14000 x G for 60 second. The supernatant was discarded and the protein pellet was dissolved with 100 µl SDS PAGE sample buffer, heated in boilling water for 5 minutes and loaded into the SDS PAGE gel (15 µl/5-mm-wide well.).

The second immunoblot was between the CNTKQTP-immunised-goat serum and recombinant haemagglutinins of all known haemagglutinins H1-H12, H14 and H15. The recombinant heamagglutinins, which were expressed in mammalian cells were purchased from Sinobiologicals Inc. China (Table 1). Each recombinant haemagglutinin was dissolved in SDS-PAGE sample buffer at 200 µg/ml, heated in boiling water for 5 minutes and loaded into the SDS PAGE gels 5 µl/3.4-mm-wide well.

Protein samples were separated on 10% separating gels then transfered onto a nitrocellulose membrane. Reversible staining with *Ponceau S* was carried out to confirm the successful transfer and to asses the amount of each haemagglutinin on the membrane (Rosenberg 1996a). After blocking with skimmed milk (5 mg/ml in what?, 2 hours), serum from the CNTKQTP-immunised goat diluted at 1 : 200 in PBS containing 5% normal

rabbit serum were added and incubated at 25°C for 2 hours. After washings four times with PBST, rabbit-anti-goat-IgG- -HRP-conjugate (Sigma Co. Singapore) diluted at 1 : 5000 was added and incubated for 2 hrs. After washing four times, chromogenic DAB (3,3'-diaminobenzidine tetrahydrochloride) substrate (H_2O_2) was added to detect bound antibody.

Sequence analysis

The alignment of CNTKCQTP epitope with the amino acid sequence of haemagglutinins used in this study was analysed using a bioinformatics software, Genious (Biomatters Ltd, Auckland, New Zealand). The amino-acid sequences of the recombinant haemagglutinins were download from Genebank (NCBI) based on the accession number provided by the vendor (Sino Biological Inc.). The similarity or percent of identity of of the CNTKCQTP epitope to its analogue in each haemagglutinin was determined by the percentage of identical amino acid.

Table 1. Recombinant Haemagglutinin (HA) from Sinobiological Inc., China used in this study

HA	Source of gene	Purity	Mr on SDS-PAGE/length	Catalog No.
H1	HA1+HA2, 2 aa deletion, uncleaved) from A/California/07/2009 (H1N1),	>95%	75-85 kDa 529 aa	11085-V08H
H2	HA1+HA2, uncleaved) from (A/Japan/305/1957(H2N2)	>97%	75-85 kDa 525 aa	11088-V08H
H3	Native, HA1+HA2, uncleaved) from A/Brisbane/10/2007(H3N2)	>97%	90-100 kDa 531 aa	11056-V08H
H4	HA1+HA2, uncleaved) from A/Swine/Ontario/01911-1/99(H4N6)	>92%	65-75 kDa 528 aa	11706-V08H
H5	cleavage site mutated (RESRRKKR→ TETR, HA1+HA2, uncleaved) from A/Indonesia/5/2005(H1N1)	>98%	70-80 kDa 531 aa	11060-V08H1
H6	HA1+HA2, uncleaved) from A/northern shoveler/California/HKWF115/2007(H6N1)	>93%	65 KDa/ 529 aa	11723-V08H
H7	Native, HA1+HA2) from A/Netherlands/219/03(H7N1)	>87%	59 kDa/ 536 aa	11082-V08B
H8	HA1+HA2, uncleaved) from A/pintail duck/Alberta/114/1979(H8N4)	>95%	53.1 kDa 529 aa	11722-V08B
H9	HA1+HA2, uncleaved) from A/chicken/Korea/164/04(H9N8)	>95%	58.2 kDa/ 523 aa	40183-V08B
H10	HA1+HA2, uncleaved) from A/duck/Hong Kong/786/1979(H10N3)	>95%	60-65 kDa/ 525 aa	11693-V08H
H11	HA1+HA2, uncleaved) from (A/mallard/Alberta/294/1977(H11N9)	>97%	65-70 kDa/ 528 aa	11704-V08H
H12	HA1+HA2, uncleaved) from A/green-winged teal/ALB/199/1991(H12N5)	>97%	80-90 kDa/ 527 aa	11718-V08H
H13	HA1+HA2, uncleaved) from A/black-headed gull/ Netherlands/1/00 (H13N8)	>95%	60-70 kDa/ 528 aa	11721-V08H
H15	HA1+HA2, uncleaved) from A/duck/AUS/341/1983(H15N8)	>97%	75 kDa/ 534 aa	11720-V08H

RESULTS AND DISCUSSIONS

Results

CNTKCQTP antibody

The titre of antibody to CNTKCQTP epitope in sera before and after vaccination with CNTKCQTP MAP is presented in Figure 1. As shown in the Figure, no antibody was detected in sera before vaccination using either CNTKCQTP MAP or GNCNTKCQTPMGAINSS single peptide as antigens. After four vaccinations the serum had a high titre of antibodies as it recognised the CNTKCQTP MAP at high 1 : 125,600 dilution. However, the titre with GNCNTKCQTPMGAINSS single peptide was much lower than the CNTKCQTP MAP. The serum could not recognize the single peptide at dilution higher than 1 : 400. This means that the titer against the MAP peptide were higher by 126600/400 = 314 times compared to that of single peptides.

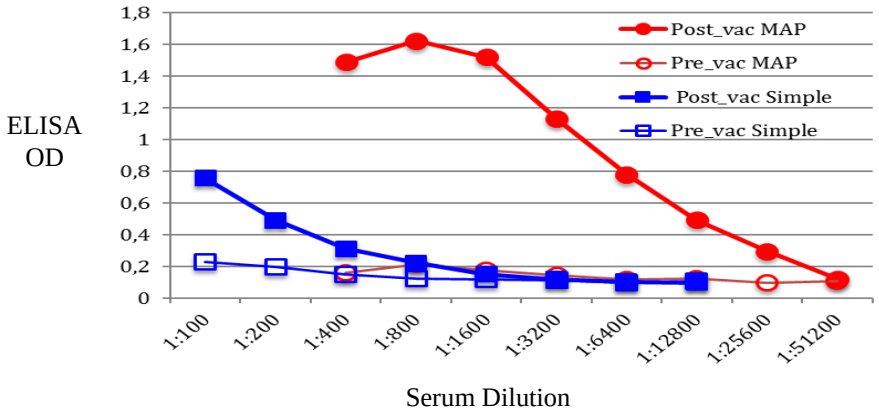

Figure 1. Antibody titre in CNTKCQTP MAP antiserum as detected in ELISA using the CNTKCQTP MAP (pre - and post- vac MAP) and GNCNTKCQTPMGAINSS single peptides (pre- and post-vac) as antigens.

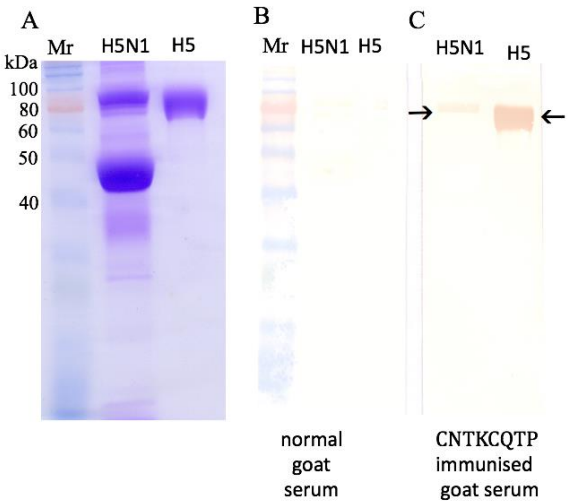

Figure 2. Reaction of CNTKCQTP MAP antisera with H5N1whole virus and recombinant H5 haemagglutinin protein in immunoblotting. H5N1whole virus proteins (H5N1) and recombinant H5 haemagglutinin (H5) separated on SDS-PAGE gel and (A) stained with Coomasie-blue. SDS-PAGE separated protein transferred to a nitrocellulose membrane and reacted with (B) normal, pre-immune goat serum (B) and (C) goat CNTKCQTP-MAP antiserum. Arrows = H5N1 haemagglutinin.

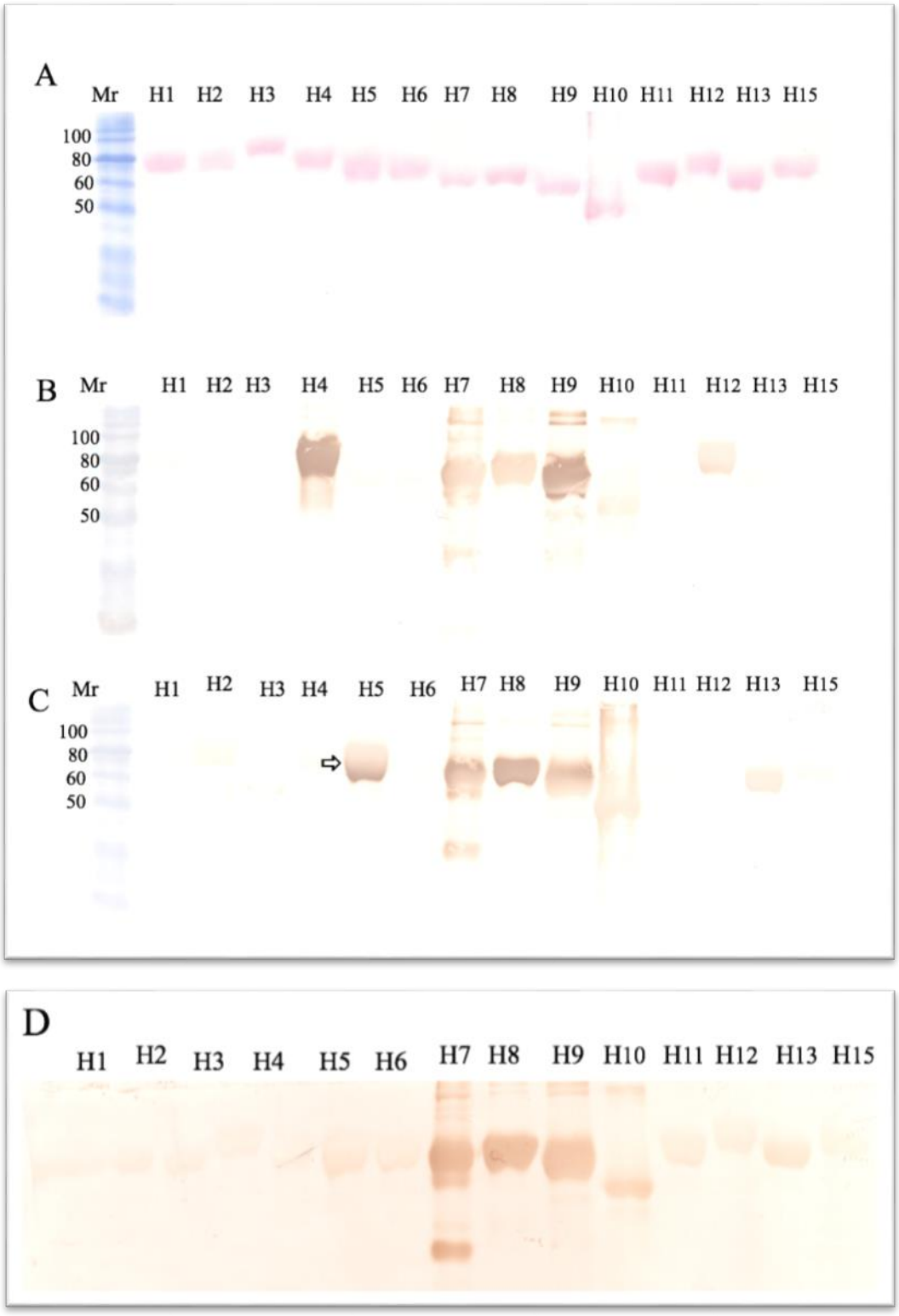

Figure 3. Reactivity of recombinant avian influenza haemagglutinins of different subtypes with serum from normal, non-immunised (B, D) and CNTKCQTP-MAP immunised goats (C). The amount of each recombinant haemagglutinin transferred onto the membrane was equal as shown by the intensity of *Ponceau*-stained band (A).

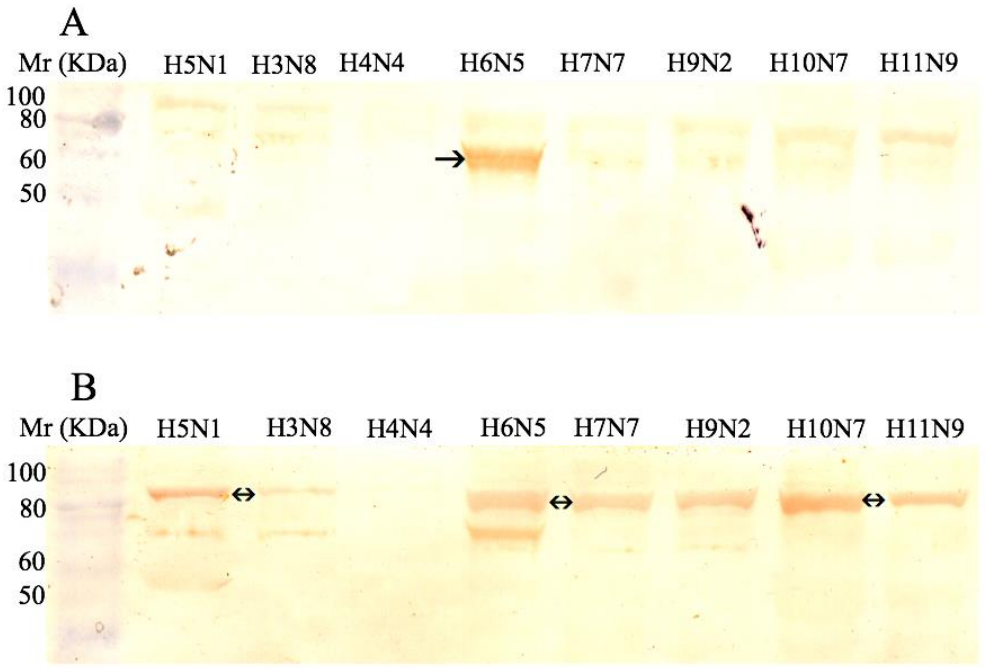

Figure 4. Reactivity of various subtypes of avian influenza virus proteins with sera from non-immune (A) and CNTKCQTP-MAP immunised goat (B). Notice that serum from non-immunised goat recognized strongly a protein band of H6N5 virus (arrow), the molecular weight of that protein was smaller than that of haemagglutinin (double head arrows).

The CNTKCQTP MAP antiserum recognised not only the peptide, but also recombinant haemagglutinin H5 and haemagglutinin protein from H5N1 virus. The antiserum recognized only one of the H5N1-virus proteins, which was supposedly haemagglutin because its molecular weight was similar to that of the recombinant H5. None of H5N1-virus proteins was recognised by the CNTKCQTP MAP antiserum (Figure 2C). Based on the thickness and stain intensity of immunoblot band, the recognition of the recombinant H5 protein by CNTKCQTP MAP antiserum was much stronger than that for H5N1-virus hemagglutinin. The differences could be attributed to the bigger amount of recombinant H5 than that of H5N1 haemagglutinin transferred onto the membrane (Figure 2A). The pre-immune serum was negative for any antibody to H5N1virus as it did not recognized any of the H5N1-virus proteins and the recombinant H5 proteins (Figure 2B).

The CNTKCQTP-MAP anti-serum recognized not only recombinant haemagglutinin H5, but also haemagglutinins H7, H8, H9, and to a lesser extend, H13 (Figure 3C). Serum from pre-immunised goat did not recognize haemagglutinin H5, but it recognised haemagglutinins H4, H7, H8, H9 and H13 (Figure 3B). Serum from a normal, non-vaccinated goat that had been kept frozen at -20^{o} for five years, also recognized haemagglutinins H7, H8, and H9 (Figure 3D).

The negative results in the immunoblot assay was convincingly attributed to the absence of binding between haemagglutinin and specific antibody in the sera. It was not due to the absence or limited amount of haemagglutinin transferred onto the membrane. Based on the thickness of *Ponceaou*-stained bands, the amount of each haemagglutinin transfered onto the nitrocellulose membrane was more or less equal (Figure 3A). The purities of the recombinant haemagglutinins which were claimed by the vendor to be >95% for most haemagglutinins, and the diversity of the molecular weights were approved by the *Ponceaou*-stained (Table 1).

When reacted with whole virus, the CNTKCQTP-MAP antiserum recognized a protein from H5N1, H3N8, H6N5, H7N7, H9N2, H10N7 and H11N9. Protein in each virus subtype recognized by the antiserum has the same molecular weight, which was about 85 kDa. Serum from normal or preimmunised goat recognised a H6N6 protein band of MW which is of smaller size than expected MW of H6N5 haemagglutinin (Figure 4, arrow).

Antibody to CNTKCQTP in chicken

The presence or absence of antibody to CNTKCQTP epitope in H5N1 immune chicken sera is presented in Figure 5. The levels of exposure of

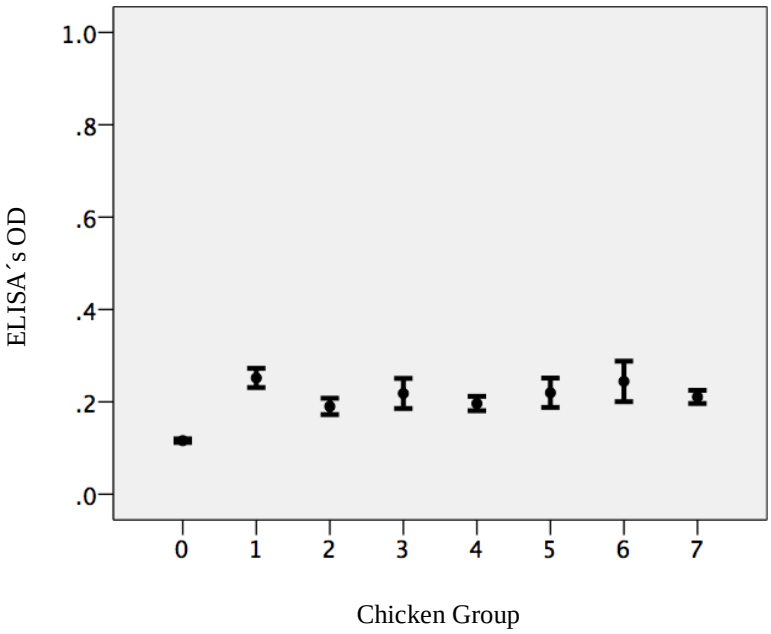

Figure 5. Antibody levels to CNTKCQTP-MAP in H5N1 immune chicken sera. 0= SPF (specific pathogen-free), 1= prevaccinated, 2= vaccinated once with killed AI vaccine (Medivac^{R}), 3= vaccinated twice, 4= vaccinated three times, 5= vaccinated then challenged with H5N1 and sera collected ≤ 7 dpi (day post infection), 6= vaccinated then challenged, sera collected 14-21 dpi, 7= vaccinated then challenged, sera collected ≥35 dpi. Bars= mean and 95% confidence interval of the means from 20 birds (groups 1-70, and from 3 birds (group 0).

Table 2. Alignment CNTKCQTP epitope with analogue epitopes in haemagglutinin

Haem-agglutinin	Subtype	Gene Bank Acc No.	Amino acid sequence	Position	Identity (%)
H1	H1N1	ACP44189.1	CNTTCQTP	292-299	87.5
H2	H2N2	AAA43185.1	CETKCQTP	288-295	87.5
H3	H3N2	ABW23353.1	CNSECITP	293-300	62.5
H4	H4N6	AAG17429.1	CVSKCHTD	291-298	50
H5	H5N1	ABW06108.1	CNTKCQTP	290-297	100
H6	H6N1	ACE81692.1	CDATCQTI	292-299	50
H7	H7N7	AAR02640.1	CEGDCYHS	293-300	25
H8	H8N4	AAG38554.1	CNTKCQTY	298-305	87.5
H9	H9N8	ABH12262.1	CVVQCQTE	286-293	50
H10	H10N3	BAF46762.1	CESKCFWR	287-294	37.5
H11	H11N9	ABB87228.1	CSTKCQSE	290-297	62.5
H12	H12N5	ABB88110.1	CVTECQLN	290-297	50
H13	H13N8	AAV91212.1	CNTKCQTS	290-297	87.5
H15	H15N8	ABB88132.1	CEGECFYS	296-303	25

chickens to H5N1 virus ranged from being anybody free as represented by SPF sera (group 0) to the those being exposed and represented by sera from chickens vaccinated multiple times and sera obtained following challenged with live H5N1 virus. The level of antibody to CNTKCQTP, as indicated by the ELISA's OD, in the SPF chicken was marginal lower compared to other groups. There were no differences in ODs between sera from pre-vaccinated (group 2) and post vaccinated birds (groups 2, 3, 4), and between pre-vaccinated (group 2) and post-challenged birds (groups 5, 6, 7). The low ELISA ODs indicated the absence of antibody to CNTKCQTP in all groups of the chicken.

Amino-acid sequence alignment

Alignment of CNTKCQTP epitope with analogue epitope in the amino acid sequence of haemagglutinins used in this study is presented in Table 2. Except for H5, none of the haemagglutinin had 100% similarity with the CNTKCQTP epitope. Four haemagglutinins (H1, H2, H8 and H13) had analogue epitopes with only one amino acid different with the CNTKCQTP epitope. The analogue epitope in the haemagglutinins H7 and H15, which had had only two identical amino acids, were the most divergent.

Discussion

This study shows that the linear CNTKCQTP epitope, which is located in the amino acid residue 274-281 of H5N1 haemagglutinin is immunogenic as high antibody titre was obtained following immunization with the CNTKCQTP MAP. The goat-CNTKCQTP-antisera would be useful as a reagent in the development of diagnostic test for H5N1 avian influenza because it recognized H5N1 haemagglutinin, either as a recombinant protein or as a native protein present in the H5N1 virus particle. The production of goat-CNTKCQTP-antisera was technically simple and inexpensive and this is important to overcome obstacles of using CNTKCQTP-monoclonal antibody that is expensive for routine diagnostic use (Velumani et al. 2011).

Interest in developing diagnostic tools for influenza has been great because the disease has been one of the most important diseases in human and animals. Point-of-care (POC) tests, most of which belong to the group of rapid immunological tests, have been the most widely developed tests because their benefits in the control of influenza either in animals or in human have been proven. In human, the ability to confirm that an illness is or is not caused by an influenza virus, using a POC test, can be a lifesaving undertaking because effective anti-influenza drugs are available (Gavin & Thomson 2003). The importance of a POC test in the control measure of HPAI has been demonstrated in the PDSR (participatory Disease Surveillance and Response) project, a successful HPAI control program in sector-4 poultry in Indonesia (Hawkes et al. 2014; Tarigan 2015).

The accuracy of an immunological test depends on the specificity of antibody used in the test. Being very specific, because it recognized only one epitope in the antigens, monoclonal antibodies have been used widely in the development of POC tests. For this reason, a large number of attempts have been made to produce monoclonal antibodies against influenza viruses (Wang et al. 2000; Rai et al. 2010; Wei et al. 2011; Bhat et al. 2013). Sometimes, however, a monoclonal antibody is too specific that it can not tolerate even a minor variation in its epitope, which reduce its diagnostic sensitivity when used in dignostic assay (Khan 2014; Singh et al. 2014).

The CNTKCQTP -polyclonal antibody produced in this study was also expected to be highly specific because its was produced against a short peptide expected to contain only a few antigenic epitopes. Additionaly polyclonal antibodies are considered to be more tolerant of antigenic variation in the epitope, and therefore when used in immunoassy, it should have higher sensitivity than monoclonal antibody.

As shown in this study, the polyclonal antibody no longer recognized the sequence (analogous epitope) even only one amino acid different, either the difference in the middle (haemagglutinins H1 and H2) or at the end of the sequence (haemagglutinins H8 and H13). This results may indicate that the CNTKCQTP-MAP used to immunised the goat contains only a single epitope.

Previous study reported that monoclonal antibody to CNTKCQTP epitope, 5F8, had high specivicity (Prabakaran et al. 2009) and when used in an epitope-blocking ELISA against recombinat H5N1 haemagglutinin (HA0), the ELISA could identify specifically chicken that had been vaccinated against H5N1 virus and no cross reactions were observed with subtypes H1-H4, H6-H16 influenza viruses.

Unlike the 5F8-monoclonal antibody, the CNTKCQTP-MAP polyclonal antibody produced in this study was not specific for haemagglutinin of H5N1, because it also recognised haemagglutinin of other AIV subtypes, including H3N8, H4N4, H6N5, H7N7, H9N2, H10N7 and H11N9.

The use of recombinant haemagglutinins in immunoblot assay in the present study failed to establish the specificity of CNTKCQTP-MAP antiserum. This was because the goat used in the antibody production already had antibody to hemagglutinin of some isolates especially H7, H8 and H9 before immunisation. It is unknown how common it is normal or non-vaccinated goats being seropositive to

H7, H8 and H9 haemagglutinins. However, the fact that serum from a non-immunised, heathy goat, collected five years ago, was also positive to those haemagglutinins when tested similarly may indicates that the condition is common. However, It is unknown whether the seropositivity was caused by infection with related subtypes of influenza-viruses or induced by other immunologically cross-reactive agents . The later possibility was more likely because it was supported by the results of other immunoblot assay using whole inactivated-influenza viruses in which the same non-vaccinated serum did not recognise haemagglutinin from H7N7 and H9N2 subtypes influenza viruses.

Before immunisation with the CNTKCQTP -MAP, the goat had been tested and it was seronegative to H5N1 virus. We did not, however, test the goat against hemagglutinin of other subtypes. In addition to our failure to envisage the possibility of the goat seropositive to haemagglutinin of other serotypes, the array of recombinant haemagglutins used in detection of the antibodies was not available untill the goat exsanguinated. This study, therefore, convinced the vital importance of testing animals to be used in the production of antiserum to be tested against haemagglutinin of subptypes all influenza virus before used.

The second aspect of the CNTKCQTP-peptide evaluated in this study, the use of the linear peptide in indirect ELISA to detect antibody, also generated results that are different to those of previous study (Velumani et al. 2011). In that study, the peptide-ELISA was able to specifically identify chicken that had been vaccinated with H5N1 virus of various clades. In the present study, on the other hand, a similar ELISA could not detect the present of specific antibody in chicken that had been vaccinated once, twice, thrice, or even in vaccinated chicken that had been challenged with a life H5N1 virus. There are three things that were different between ELISA in this study and that in previous study. First, the concentration of peptide used to coat the microtitre plate was 5 µg/ml in this study and 10 µg/ml in previous one. We are convinced that this concentration difference could not attributed to differences in the results because in our preliminary experiment, we found that no differences between both concentrations (data not presented). Second, in this study non-fat-skimmed milk was used as a blocking reagent whereas in previous study, bovine serum albumin was used. In our previous study, we found that non-fat-skimmed milk was effective as blocking agent and therefore the blocking reagent could not also be attributed to the differences result of this study and previous study (Tarigan et al. 2015). Third, in this study the chicken were immunised with a commercial H5N1 vaccine, whereas, in previous study the vaccine was prepared by the authors themselves. Because the vaccine was prepared by the authors themselves, the purity and the amount of immunogen contained in the vaccine were known with certainty. The purity and the amount of immunogen in the commercial vaccine in the present study were unknown.

In summary, immunisations of goat with the CNTKCQTP epitope, in the form of MAP, produced antibody that recognised H5 haemagglutinin but only H5N1 virus. The antibody is useful as the main reagent in development of rapid test for H5N1 avian influenza. Some apparently pre-immunised goats are seropostive for some influenza haemagglutinins, therefore testing goats against all influenza haemagglutinins before used for antibody production is vitally important. This study fail to demonstrate the presence of antibody to CNTKCQTP epitope in chicken that had been vaccinated or infected with H5N1 virus.

CONCLUSION

High titre antibody to CNTKCQTP epitope was produced by immunising goat with CNTKCQTP MAP. The serum recognised haemagglutinin H5 and therefore is useful as a reagent for detection of H5N1 virus. However, unlike CNTKCQTP monoclonal antibody generated previously, the serum is not specific for H5N1 haemagglutinin as it also recognises haemagglutinin of other influenza virus subtypes. Some apparently pre-immunised goats are seropostive for some influenza haemagglutinins, therefore testing goats against all influenza haemagglutinins before used for antibody production is vitally important. ELISA using peptide containing the epitope sequence, GNCNTKCQTPMGAINSS, cannot be used to detect anti-H5N1 antibody in chicken immunised with commercial-H5N1 vaccine.

ACKNOWLEDGMENT

Some apparently pre-immunised goats are seropostive for some influenza haemagglutinins, therefore testing goats against all influenza haemagglutinins before used for antibody production is vitally important.

This work was supported by the Australian Centre for International Agricultural Research under Grant AH/2010/039. The authors thank Mrs Gita Sekarmila, Mr Achpas and the animal caretakers for their excellent technical assistance.

REFERENCES

Bhat S, Bhatia S, Sood R, Bhatnagar H, Pateriya A, Venkatesh G. 2013. Production and characterization of monoclonal antibodies against nucleoprotein of avian

influenza virus. Monoclon Antib Immunodiagn Immunother. 32:413-418.

Claas EC, de Jong JC, van Beek R, Rimmelzwaan GF, Osterhaus AD. 1998. Human influenza virus A/HongKong/156/97 (H5N1) infection. Vaccine. 16:977-978.

[FAO] Food and Agriculture Organization. 2011. Approaches to controlling, preventing and eliminating H5N1 Highly Pathogenic Avian Influenza in endemic countries. FAO Animal Production and Health Paper No. 171.

[FAO] Food and Agriculture Organization. 2012. H5N1 HPAI global overview, January–March 2012. Issue No. 31. [accessed April 16th 2015]. http://www.fao.org /docrep/015/an388e/an388e.pdf

Gavin PJ, Thomson RB. 2003. Review of rapid diagnostic tests for influenza. Clin Appl Immunol Rev. 4:151-172.

Hawkes P, Echalar R, Budiharta S, Soenarjo S. 2014. USAID/Indonesia Avian And Pandemic Influenza (API) program evaluation: 2009–2014. GH Tech Project Bridge IV, Report No. 14-B4-009.

Ho HT, Qian HL, He F, Meng T, Szyporta M, Prabhu N, Prabakaran M, Chan KP, Kwang J. 2009. Rapid detection of H5N1 subtype influenza viruses by antigen capture enzyme-linked immunosorbent assay using H5- and N1-specific monoclonal antibodies. Clin Vaccine Immunol. 16:726-732.

Khan FH. 2014. Antibodies and their applications. In: Verma AS, Singh A, editors. Animal biotechnology-model in discovery and translation. Amsterdam (NL): Acad Press. p. 473-490.

Lin J, Wang R, Jiao P, Li Y, Li Y, Liao M, Yu Y, Wang M. 2015. An impedance immunosensor based on low-cost microelectrodes and specific monoclonal antibodies for rapid detection of avian influenza virus H5N1 in chicken swabs. Biosens Bioelectron. 67:546-552.

Prabakaran M, Ho HT, Prabhu N, Velumani S, Szyporta M, He F, Chan KP, Chen LM, Matsuoka Y, Donis RO, Kwang J. 2009. Development of epitope-blocking ELISA for universal detection of antibodies to human H5N1 influenza viruses. PLoS One. 4:e4566.

Rai M, Bhatia S, Malik YP, Dubey SC. 2010. Production and characterization of monoclonal antibodies against NS1 protein of H5N1 avian influenza virus. Hybridoma (Larchmt). 29:183-186.

Rosenberg IM. 1996a. Protein analysis and purification. benchtop techniques. Boston (USA): Birkshauser Boston. p. 158-159.

Rosenberg IM. 1996b. Protein analysis and purification. benchtop techniques. Boston (USA): Birkhauser Boston. p. 130.

Sims LD, Domenech J, Benigno C, Kahn S, Kamata A, Lubroth J, Martin V, Roeder P. 2005. Origin and evolution of highly pathogenic H5N1 avian influenza in Asia. Vet Rec. 157:159-164.

Singh A, Chaudhry S, Agarwal A, Verma AS. 2014. Antibodies: Monoclonal and Polyclonal. In: Verma AS, Singh A, editors. Animal biotechnology-model in discovery and translation. Amsterdam (NL): Acad Press. p. 265-287.

Tarigan S. 2016. Peranan Point-of-Care-Test dalam pengendalian High Pathogenic Avian Influenza di Indonesia. Wartazoa. 26:39-50.

Tarigan S, Indriani R, Durr PA, Ignjatovic J. 2015. Characterization of the M2e antibody response following highly pathogenic H5N1 avian influenza virus infection and reliability of M2e ELISA for identifying infected among vaccinated chickens. Avian Pathol. 44:259-268.

Velumani S, Ho HT, He F, Musthaq S, Prabakaran M, Kwang J. 2011. A novel peptide ELISA for universal detection of antibodies to human H5N1 influenza viruses. PLoS One. 6:e20737.

Wang X, Castro AE, Castro MD, Lu H, Weinstock D, Soyster N, Scheuchenzuber W, Perdue M. 2000. Production and evaluation criteria of specific monoclonal antibodies to the hemagglutinin of the H7N2 subtype of avian influenza virus. J Vet Diagn Invest. 12:503-509.

Wei J, Yan B, Chen Z, Li T, Deng F, Wang H, Hu Z. 2011. Production and characterization of monoclonal antibodies against the hemagglutinin of H5N1 and antigenic investigation of avian influenza H5N1 viruses isolated from China. Can J Microbiol. 57:42-48.

AUTHOR GUIDELINES

Indonesian Journal of Animal and Veterinary Sciences, or IJAVS contains:

(i) Primary scientific manuscript of unpublished research results.
(ii) Elucidation of research methodes and innovative techniques which is useful for research development.

AUTHOR GUIDANCE

Manuscript is written in good English, accompanied with abstract in English and Indonesian. Manuscript is typewritten on the A4 paper size with 2 spaces distance and 4 cm from left side, 3 cm from right side, 3 cm from top and buttom sides. We provide you with IJAVS Template that you can find in our website: http://medpub.litbang.pertanian.go.id/index.php/jitv.

SCRIPTWRITING SYSTEMATICS

1. **Title:**
   Should be comprehensive, but it is made as short as possible. Subtitle can be given if it needed.

2. **Name and Address of Author:**
   Author's name is written completely (without degree) and typewritten by CAPITAL letter. If the author is more than 1 person with different address, Arabic numbers superscript shouk be given behind each name. Author's address written under author's name, consisting of institution name and its complete address, made in line with number of index on behalf of the author and typewritten by ITALIC.

3. **Abstract:**
   Abstract is gift of manuscript, written in Indonesian or English, do not more than 250 words and stated in one paragraph. Abstract consists of backgound, purpose, material and methode, result and conclution. The author's name (in CAPITAL form), publication year, manuscript title and journal name are listed before abstract content with layout as reference. Keywords are listed under the abstract, maximum 5 words.

4. **Introduction:**
   Is consisting of research background, issue, efforts which have been made, approach taken to solve the problem and research purpose.

5. **Material and Method:**
   Elucidating clearly about materials used and method carried out. If the material using animals in the experiment, please indicate that the animals are performed according to animal ethics and welfare. See ethical statement in the attachment.

6. **Result and Discussion:**
   It presents and discuss clearly and completely achieved research results based on the purpose. Result and discussion may be presented separated or united. Result description may be completed by consise tables and cleare ilustrations (black and white graphics, figures or photos) on separated page. Tabel description (on top) and ilustration (in bottom) should be clear and independent, so readers may easily understand the table without read the text. Discussion description consists of description of result and research mean and benefit associated with issue which will be solved. Measurement units both in table or ilustration use metric system.

7. **Conclusion:**
   It is a manuscript final summary.

8. **Acknowledgement:**
   It can be written if needed.

9. **References:**
   The author is recommended to use Mendeley Program (http://www.mendeley.com) and citation style of Taylor & Francis - Council of Science Editors (author-date). Mendeley program utilization is aimed to avoid mistakes in citations and references writting. Cited references (preferebly, 80% is primary article and the last 10 years publication). and should not from unpublished articles such as practical guidance and research report, except thesis and dissertation. Download is allowed if it is from electronic magazine, genom database or patent.

   Citation in the references:
   Literatures in reference are written alphabetically based on the author's name. Same author is written sequentially starting from earlier order.

   Example of reference writing

   Primary paper:
   Bhanja SK, Anjali DC, Panda AK, Sunder GS. 2009. Effect of post hatch feed deprivation on yolk-sac utilization and young broiler chickens. Asian-Aust J Anim Sci. 22:1174-1179.

   Book:
   a. Lawrence TLJ, Fowler VR. 2002. Growth of farm animals. 2nd ed. New York (USA): CABI Publishing.
   b. Bamualim A, Tiesnamurti B. 2009. Konsepsi sistem integrasi antara tanaman padi, sawit, dan kakao dengan ternak sapi di Indonesia. In: Fagi AM, Subandriyo, Rusastra IW, penyunting. Sistem integrasi ternak tanaman padi, sawit, kakao. Jakarta (Indones): LIPI Press. p. 1-14.
   c. Paloheimo M, Piironen J, Vehmaanpera J. 2010. Xylanases and cellulases as feed additives. In: Bedford MR, Partridge GG, editors. Enzymes in farm animal nutrition. 2nd ed. New York (USA): CABI Publishing. p. 12-53.

Proceeding:
Umiyasih U, Antari R. 2011. Penggunaan bungkil inti sawit dan kopra dalam pakan penguat sapi betina berbasis limbah singkong untuk pencapaian bobot badan estrus pertama >225 kg pada umur 15 bulan. Prasetyo LH, Damayanti R, Iskandar S, Herawati T, Priyanto D, Puastuti W, Anggraeni A, Tarigan S, Wardhana AH, Dharmayanti NLPI, editors. Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner. Bogor (Indones): Pusat Penelitian dan Pengembangan Peternakan. p. 192-199.

Thesis:
Krisnan R. 2008. Kombinasi penggunaan probiotik mikroba rumen dengan suplemen katalitik pada pakan domba (Tesis). [Bogor (Indones)]: Institut Pertanian Bogor.

Electronic magazines:
Wina E, Tangendjaja B, Dumaria. 2008. Effect of *Calliandra calothyrsus* on *in vitro* digestibility of soybean meal and tofu wastes. Livest Res Rural Develop. Vol. 20 Issue 6. http://www.lrrd.org/lrrd20/6/wina 20098.htm.

Institution:
a. [NRC] National Research Council. 1985. Nutrient requirements of sheep. 6th revised. Washington DC (USA): National Academic Press.
b. [CDC] Centers for Disease Control. 2006. Standard operating procedure for the direct Rapid Immunohistochemistry Test (dRIT) for the detection of rabies virus antigen. [accessed December 20th 2011]. http://www.rabiesblue print.com/IMG/pdf/DRIT_SOP.pdf.

Patent:
Blanco EE, Meade JC, Richards WD. 1990. Ophthalmic ventures, assignee. Surgical stapling system. United States patent US 4,969,591. 1990 Nov 13.

10. **Citation in text:**
Citation consists author's last name and publication year.

Example:

a. One author: ..... grow slower than lamb fed cattle's milk (Supriyati 2012). Supriyati (2012) formulates.....
b. Two authors: ..... expect, end maintenance weight (Khasrad & Rusdimansyah 2012). Khasrad & Rusdimansyah (2012) argued.....
c. Three authors or more: ..... based on DNA mitochondria analysis (Mtileni et al. 2011). Mtileni et al. (2011) reports.....
d. Same author cited from 2 different papers: (Purwadaria et al. 2003a, 2003b).
e. Author with same family name is written consecutive: (Dawson J 1986; Dawson M 1986).
f. Several different authors are written consecutive: (Kannan et al. 2000; Grandin 2007; Santosa et al. 2012).
g. Institution: BPS (2011).....

11. **Table:**
a. Standard word used is Times New Roman with 1 space distance and 11 of font size.
b. Title is simple, clear, and understable sentence without reads the manuscript.
c. Each colum from table should has headling. Its unit separated from title by coma, in parentheses, or at its bottom.
d. Table description is written under the table with 1 space distance and 11 of font size. Data source is written under the table or in the table in own header.

Dividing line is made in form of horizontal.

12. **Figure and graphic:**
a. Title uses Times New Roman with 1 space distance and 11 of font size. It is a simple and clear sentence which is laid under the figure or graphic.
b. Line in graphic should show clearly difference of one and others, if there is more than one curve.
c. Clear contrast figure with proportionate size and high resolution to present the best performance.

Write figure or graphic source under the title.

1. If written manuscript is more than one, it needed an approval from the other authors by enclose initial behind each name.
2. Complate manuscript is sent in three copies to Editorial Board of IJAVS and its electronic file, or by online: http://medpub.litbang.pertanian.go.id/ index.php/jitv

The author is entitled to 1 original journal and 10 its reprints.

Jurnal Ilmu Ternak dan Veteriner

IJAVS Indonesian Journal of Animal and Veterinary Sciences

Center for Animal Research and Development
Indonesian Agency for Agricultural Research and Development
Padjajaran St. Kav. E59, Bogor 16128
Phone: 0251 - 8322185 | Fax: 0251 - 8380588
e-mail: jitvnak@yahoo.com/jitvnak@litbang.pertanian.go.id
http://medpub.litbang.pertanian.go.id/index.php/jitv/index

Dear
Editorial Board of Indonesian Journal of Animal and Veterinary Sciences
Indonesian Center for Animal Research and Development
Padjajaran St. Kav. E59, Bogor 16128

ETHICAL STATEMENT

Respect to paper submission to Indonesian Journal for Animal and Veterinary Science, by following this letter, I here:

Name :
Institution :
Title of Paper :

Acknowledging that the paper submitted is my own or team work, that:

- ☐ It is original or free from: a) fabrication; b) falsificasin; c) plagiarism; d) duplication; e) fragmentation; and f) data/content copyright infringement.
- ☐ It is obtained through **true** scientific meeting or free from: a) engineered scientific meeting; and b) not attended meeting.
- ☐ It is ensure the studies involving animals that are performed according to animal ethics and welfare.
- ☐ It is unpublished in other publications.

This acknowledgment is made honestly and responsible based on Regulation of Head of Indonesian Institute of Science Number 06/E/2013 about Code of Ethic of Researcher.

, 2016

Applicant,

_____

Author's colleague:

Name	Sign

Note:
Please sent statement letter with original signed and stamped **by post** to:
Technical Editor of Indonesian Journal of Animal and Veterinary Sciences
Pajajaran St. Kav. E59 Bogor 16128. Phone: (0251) 8322185 Fax. (0251) 8380588
Email: jitvnak@yahoo.com/jitvnak@litbang.pertanian.go.id
Website: http://medpub.litbang.pertanian.go.id/index.php/jitv/index

Jurnal Ilmu Ternak dan Veteriner

IJAVS *Indonesian Journal of Animal and Veterinary Sciences*

Indonesian Center for Animal Research and Development
Indonesian Agency for Agricultural Research and Development
Padjajaran St. Kav. E59, Bogor 16128
Phone: 0251 - 8322185 | Fax: 0251 – 8380588
e-mail: jitvnak@yahoo.com/jitvnak@litbang.pertanian.go.id
http://medpub.litbang.pertanian.go.id/index.php/jitv/index

COPYRIGHT TRANSFER FORM

Title of Paper :

Author :

This paper is original and the author diverts its copyright to Indonesian Journal of Animal and Veterinary Sciences, incase if and when this paper is accepted.

Everyone listed as author in this paper had contributed to substation and intellectual and should be responsible to public. In case is notified a copyright infringement, it is responsible to the author, not responsible to Indonesian Journal of Animal and Veterinary Science.

This paper content is unpublished before and not being considered to be published in other journals.

, 2016

Approved by

Primary Author

Author's colleague:

Name	Sign

This form should be signed by **all authors and returned to the Editorial Board**. The form may be sent by post or email.

Acknowledgement

Editorial board and executive editor of Indonesian Journal for Animal and Veterinary Science (IJAVS) extent high appreciation to the expertises of peer reviewer of IJAVS (Volume 21 No. 1 2016).

1.	Prof. Dr. Abubakar	:	Postharvest Agriculture Technology - ICAPRD
2.	Prof. Dr. drh. Retno D. Soejoedono, M.S.	:	Microbiology - Bogor Agricultural University
3.	Dr. drh. Michael Haryadi Wibowo, M.P.	:	Microbiology - Gadjah Mada University
4.	Rintis Noviyanti, Ph.D.	:	Biomedical Science – Eijkman Institute

We hope this good collaboration would be continued in the future in improving IJAVS quality.

Jurnal Ilmu Ternak dan Veteriner

IJAVS Indonesian Journal of Animal and Veterinary Sciences

Volume 21, Number 1, March 2016 ISSN 0853-7380 E-ISSN 2252-696X

LIST OF CONTENT

Registered in: