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PREFACE

Research findings would be beneficial if they are presented and widely distributed to wider audiences. This journal brings you the latest research results in animal production and veterinary technologies from Egypt and Indonesia.

In this edition, volume 20 no. 3 September 2015, we proudly present articles from various disciplines such as Animal breeding; food technology; feed technology; forages technology; and veterinary technology. The articles published in this edition are: "Multi-trait and multi-source selection indices for milk production and reproductive traits in a herd of Holstein cattle in Egypt"; "Heritability estimation and non-genetic factors affecting production traits of Indonesian Ongole cross"; "Antimicrobial and anti-oxidative activities of peptides from goat milk hydrolyzed with various protease"; "Effect of enzyme supplementation on nutritive values of fermented palm kernel cake used to substitute soybean meal in broiler diet"; "Administration of extract *Salix tetrasperma* combined with turmeric and neem extract to improve eggs quality of chicken reared under heat stress"; "Lipid profiles of blood serum and fatty acid composition of meat of hybrid duck fed diet with Noni (*Morinda citrifolia*) fruit meal"; "Production and quality of *Murdannia bracteata* biomass as impact of magnesium foliar fertilizer"; "Pathological changes of suspected tetrachloro dibenzo-ᵽ-dioxins/tetrachloro dibenzofurans toxication in beef cattle" and "Circulating H5N1 virus among native chicken living around commercial layer farms".

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.

Bogor, September 2015

Chief Editor

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Multi-Trait and Multi-Source Selection Indices for Milk Production and Reproductive Traits in a Herd of Holstein Cattle in Egypt

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ABSTRAK

Faid-Allah E. 2015. Indeks seleksi *multi-trait* dan *multi-source* untuk sifat-sifat produksi susu dan reproduksi pada sapi Holstein di Mesir. JITV 20(3): 159-167. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1182

Penelitian ini dilakukan dengan tujuan mencari kemungkinan meningkatkan produksi susu dan reproduktifitas sapi Holstein melalui pemanfaatan metode indeks seleksi yang meliputi indeks berikut: umum, tidak lengkap, sub dan multi informasi (*Own-Performance, Full-Sibs and Half-Sibs*). Data diperoleh dari peternakan komersial (Safi Masr for Developing the Animal Resources), berlokasi di Delta sungai Nil, Dakahlia, Mesir. Data meliputi 4791 catatan dari 1797 ekor sapi, 794 induk dan 67 pejantan yang mewakili catatan pada kurun waktu 2002 sampai 2012. Estimasi parameter genetika dan phenotipik untuk penelitian trait/sifat dihitung dan digunakan untuk membentuk 18 indeks seleksi guna meningkatkan produksi susu dan reproduksi. Indeks penuh melibatkan produksi susu 305 hari (305-dMY), periode laktasi (LP), days open (DO) dan umur beranakn pertama (AFC) mempunyai korelasi paling tinggi dengan nilai aggregate breeding (R_{ih} = 0.518; RE=100%). Korelasinya berada pada 0,455 bila 305-dMY dihilangkan dari index. Index umum mempunyai pendugaan genetic yang maximum pada 305-dMY (132.6 kg) per generasi diikuti dengan menurunnya LP (-4,679 hari), DO (-3.449 day) dan AFC (-1,41 bulan) jika ke empat sifat dimasukkan ke dalam index (I_1). Pendugaan genetik untuk 305-dMY menurun sampai 26,84kg/generasi bila 305-dMY dihilangkan dari dari index 5 (I_5). Selanjutnya menggunakan informasi multi-sumber akan meningkatkan korelasi dengan nilai *aggregate breeding* (R_{ih}= 0.740; RE=142.91%) dan meningkatkan pendugaan peningkatan genetik tiap generasi untuk 305-dMY (209 kg) dan menurunkan pendugaan peningkatan genetik untuk LP (-6,37 hari), DO (-4,244 hari) dan AFC (1,843 bulan) apabila keempat trait/sifat dimasukkan kedalam index (I_{16}). Dapat disarankan untuk menggunakan indeks yang lebih tinggi untuk Rih ($I_{1\ (RE=100)}$) untuk meningkatkan produksi susu dan sifat reproduksi pada sapi Holstein berdasarkan strategi performannya sendiri dan menggunakan ($I_{16\ (RE=142.91)}$) berdasarkan strategi multi-sumber untuk mendapatkan akurasi yang tinggi dan perubahan genetik harapan yang tinggi per generasi dibandingkan dengan indeks general.

Kata Kunci: Bobot Badan, Parameter Genetik, *Selection Index*, Sapi Holstein

ABSTRACT

Faid-Allah E. 2015. Multi-trait and multi-source selection indices for milk production and reproductive traits in a herd of Holstein cattle in Egypt. JITV 20(3): 159-167. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1182

The main aim of this study was explore possibility to improve milk production and reproductive traits of Holstein cattle via selection index method which include general, reduced, sub and Multi-source of information indices (Own-Performance, Full-Sibs and Half-Sibs). Data was obtained from a commercial farm (Safi Masr for Developing the Animal Resources), located in the Nile Delta, Dakahlia, Egypt. Data included 4791 records of 1797 cows, 794 dams and 67 sires that represented the period from 2002 to 2012. Estimates of genetic and phenotypic parameters for studied traits were computed and used to construct 18 selection indices to improve milk production and reproductive traits. Full index incorporating milk yield at 305d (305-dMY), lactation period (LP), days open (DO) and age at first calving (AFC) had the highest correlation with aggregate breeding value (R_{ih} = 0.518; RE=100%). The correlation fell to 0.455 when 305-dMY was omitted from the index. The general index has the maximum expected genetic gain in 305-dMY (132.6 kg) per generation were accompanied by decrease of LP (-4.679 day), DO (-3.449 day) and AFC (-1.41 month) when all four traits were included in the index (I_1). The expected genetic gain for 305-dMY decreased to 26.84 kg/generation when 305-dMY was excluded in index 5 (I_5). In addition, Using multi-source of information will enhance correlation with aggregate breeding value (R_{ih}= 0.740; RE=142.91%) and raised the expected genetic gain per generation for 305-dMY (209 kg) and decreasing the expected genetic gain for LP (-6.37 day), DO (-4.244 day) and AFC (1.843 month) when all four traits were included in the index (I_{16}). It could be suggested using the higher indexes of Rih ($I_{1\ (RE=100)}$) to improve milk production and reproductive traits in Holstein cattle under own-performance strategy and using ($I_{16\ (RE=142.91)}$) under multi-source strategy to get high accuracy and higher expected genetic changes per generation compare to general index.

Key Words: Body Weight, Genetic Parameter, Selection Index, Holstein Cattle

INTRODUCTION

Breeding programs are basically designed to identify superior genotypes for different traits of economic interest, based on performance information of animals and their relatives, in order to disseminate their genes in the population. Literature shows that the implementation of selection indexes was an important step in the evolution of the dairy industry in the developed countries (Cardoso et al. 2014).

Increasing use of selection indices and greater scope in number of traits has been observed in dairy cattle populations in the past two decades included main components related to production, durability, health and reproduction in each selection index (Miglior et al. 2005). The traits that were considered for selection were milk yield, daily gain, weaning weight, calving interval, milk fat yield, productive lifetime, pre-weaning survival rate, post-weaning survival rate and age at first calving. Age at first calving and calving interval are important because they determine the days a cow is in milk and the number of calves in the productive lifetime for replacement or sale (Wahinya et al. 2015).

Multiple trait selection index is widely accepted as the method of choice when improvement is desired for more than one trait (Banga 2009). Undesirable effects were observed on traits with unfavorable correlations with milk production, such as decline in fertility. As information on other traits related to health, fertility and longevity started being recorded and genetic evaluations for these traits were performed, they were gradually included as breeding goals of dairy cattle (Norman et al. 2010).

This study was carried out to investigate the possibility to improve milk production and reproductive traits of Holstein cattle via selection index method under two strategies, own-performance strategy to use easy index and multi-source of information strategy to get high accuracy and higher expected genetic changes per generation compare to general index when more information is used.

MATERIALS AND METHODS

Data, Feeding and management: Data of Holstein cattle were obtained from a commercial farm (Safi Masr for Developing the Animal Resources), located at the Nile Delta, Dakahlia, Egypt. Data were comprised of 4791 records of 67 sires and 794 dams from the year 2002 to 2012. Genetic and non-genetic factors as sire, parity (1^{st} to $\geq 6^{th}$), year of calving (2002 to 2012) and calving season (winter from 22/12 to 21/3, spring form 22/3 to 21/6, summer from 22/6 to 21/9 and autumn from 22/9 to 21/12). Animals were housed free in shaded open yards, grouped according to average daily milk yield, and fed on TMR system a round year as recommended by NRC (2001). Holstein heifers were artificially inseminated (imported semen of Holstein sires) for the first time when reaching 350:370 kg of weight and pregnancy was detected by rectal palpation at 60 days after service. The cows were machine milked three times per day. Studied traits are 305-day milk yield (305-dMY) and lactation period (LP) as milk production traits and days open (DO) and age at first calving (AFC) as reproductive traits were expressed in time intervals. These aspects were discussed by Faid-Allah (2015).

Genetic parameters: The genetic parameters were estimated by derivative free REML with a simplex algorithm using the Multiple Trait Derivative-Free Restricted Maximum Likelihood (MTDFREML) program of Boldman et al. (1995). The animal model in matrix notation as follow**:**

$$Y = Xb + Za + e$$

where:

Y = Vector of observations (milk production and reproductive traits)

b = Vector of fixed effects (i.e. parity, year and season of calving)

a = Vector of random additive genetic direct effects (i.e. sire and dam)

X, Z = Known incidence matrices relating observations to the respective traits

e = Vector of residual effects (0, $I\sigma_e^2$)

Selection Index: The four traits studied were used in combinations to construct 18 selection indexes grouped under two strategies based on (305-dMY, LP, DO and AFC) as follows: Strategy 1: own-performance. Strategy 2: Multi-source of information (Own-Performance, Full-Sibs and Half-Sibs). The Selection criterion and the selection objectives are the same.

General Selection Index: Selection Index Program (Wagenaar et al. 1995) and Matlab program (Matlab 2002) were used to construct the selection indices. Studied traits were used to construct 18 selection indices. Selection index was obtained by solving the following equation:

$$I = b_1P_1 + b_2P_2 + \cdots b_nP_n = \sum_{i=1}^{n} biPi$$

where:

I = Selection index

bi = Index weights for each trait in the index

P_i = Phenotypic measurement for each trait in the index

The general index was obtained by solving the following equations given in matrix expression according to Cunningham (1969):

$$P*b = G*a \quad \text{to give} \quad b = P^{-1} *Ga$$

where:
P = Phenotypic variances (cov.) matrix
G = Genetic variances (cov.) matrix
a = Economic weights column vector
b = Weighting factors column vector

Reduced selection index: The reduced selection index can be developed by omitting one or more traits from the original index. In relation to the original index the efficiency of the new index, the reduced one, is expected to be decreased depending on the value of the omitted trait in the original index.

Sub-selection index: The sub-index of each trait was achieved by solving the following equation according to Cunningham (1969):

$$b = P^{-1} G$$

where:
b = Weighting factors vector for the sub index
P^{-1} = P matrix inverse
G = Covariance vector between the variables in the index and the main trait in the aggregate genotypes

Multi-Source selection index: The sources of information used in different combinations for each trait were individual's own phenotypic value (OP), its full (FS) and half sibs (HS) averages. The general outline of the selection indices for ranking of the breeding value was as follow:

$$I = \sum_{i=1}^{n} [b_{i1}(P_{i1}-\mu_i) + b_{i2}(P_{i2}-\mu_i) + b_{i3}(P_{i3}-\mu_i) + b_{i4}(P_{i4}-\mu_i)]$$

where:
n = Number of the traits
μ_i = Population mean of the i *th* trait
bij = Partial regression coefficients of i *th* trait of j *th* group of relatives (i=1-4) ; (j=1-3). P_{i1}, P_{i2}, P_{i3} and P_{i4}= OP and its FS and HS averages of animal candidates

The P and G matrices, respectively consisting of the variances (cov.) from OP, FS and HS family sources were obtained for animals. Estimation of genetic and phenotypic variances (cov.) for FS and HS performance for the P and G matrix were estimated according to the procedures given by Liljedahl et al. (1979) as follow:

σp_{ij} pi' j'= C1 $\sigma p_i p_{i'}$+ C2 $\sigma_{AiAi'}$ for P matrix
σp_{ij} Ai = C$\sigma_{AiAi'}$ (i = i' or i ≠ i') for G matrix

where:
σp_{ij} = Phenotypic variances (cov.) between traits in i and **i'** (i = i' or i ≠ i')
$\sigma_{AiAi'}$ = Additive genetic variances (cov.) or between traits in i and i' (i = i' or i ≠ i')

The procedures for obtaining C1 and C2 values for each element in P matrix and C values for each element in G matrix have been utilized according to Liljedahl et al. (1979).

Properties of the selection index: the properties of the selection index according to Cunningham (1969) were calculated as following:

1. Standard deviation of the index (σi) = $\sqrt{b'Pb}$
2. Standard deviation of the aggregate genotype (σt) = $\sqrt{a'Ga}$
3. Correlation between the index and the aggregate genotype (R_{IH})= $\sigma i/\sigma t$
4. Value of each trait in the index = Vt

$$Vt = 100 - \sqrt{\frac{b'Pb - b_i / W_{ii}}{b'Pb}} \times 100$$

where:
Vt = Value of each trait in the index
P = Phenotypic variances (cov.) matrix
B = Weighting factors column vector
w_{ii} = a diagonal element of p^{-1}

Expected genetic change ΔG (EG): EG for each trait, after one generation of selection on the index (*i* = 1) was obtained by solving either of the following equations (Van der Werf & Goddard 2003):

$$\Delta Gi = (i\ b'\ Gi)/\sigma i$$

where:
i = Selection intensity
σI = Standard deviation of the index
Gi = the i^{th} column of the G matrix

The relative economic value (Rev): The economic values (a) were calculated as one phenotypic standard deviation (σp) as relative economic weight of each trait as reported by Atil (2006) and Faid-Allah & Ghoneim (2012) as shown in table 1. It is Non-objective methods with modification in its charge to be negative for LP, DO and AFC to get higher desired genetic gain for traits under selection depends on the trait phenotypic dispersion.

RESULTS AND DISCUSSION

Descriptive statistics

Table (1) shows the arithmetic mean of milk production traits as 305 day milk yield and lactation period are 6384.95 kg and 332 day, respectively.

The average and coefficient of variability (CV %) of 305-day milk yield for Holstein cows were 4295 (CV=19.7), 9038 kg (CV=13.1) and 8455.4 (CV= 18.2) in Egypt as reported by Ashmawy & Khalil (1990), Salem et al. (2006) and Hammoud (2013), respectively.

Table 1. Descriptive statistics of studied traits for milk production and reproductive status in Holstein cows

Traits	Records №	Mean	SD	CV (%)	#Rev
Milk production traits					
Milk yield at 305d (305-dMY), kg	4791	6384.95	1236.9	19.37	1
Lactation period (LP), day	4791	332.00	49.38	14.87	-25.05
Reproductive traits (Time intervals)					
Days open (DO), day	3108	157.93	35.72	22.62	-34.61
Age at first calving (AFC), month	2431	30.51	5.12	16.79	-8.05

#Rev= the relative economic value

The lactation period (LP) for Holstein cows was found to vary from 286 to 407 days and it's CV ranged from 5 to 31.74 % as mentioned by El-Arian et al. (2003), Salem et al. (2006), Hammoud (2013) and Osman et al. (2013a) in Egypt.

Usman et al. (2012) reported that LP of Holstein cows ranged from 185 to 514 days with mean of 366.5±76.71 days (CV= 20.93).

The mean (CV %) of reproductive traits as days open and age at first calving (Table 1) are 157.93 day (22.62) and 30.51 month (16.79), respectively. The low age at first calving in a particular dairy cattle herd is a reflection of the good managerial strategy adopted in that herd. High level of management allows the growing heifers to reach the suitable body weight for breeding earlier and this in turn leads to lower age at first calving.

Table (1) shows the mean of 305-dMY were lower than those found by Abou-Bakr et al. (2006) being 10847 kg, respectively and those reported by Salem et al. (2006) being 9038 kg, respectively Holstein cows in Egypt. The mean of LP was lower than the mean of 370 and 407 days obtained by Abou-Bakr et al. (2006) and Salem et al. (2006), respectively. The estimated of DO obtained in this study was shorter than that of 255 days found by Abou-Bakr et al. (2000), but was similar to 154 days obtained by Abou-Bakr et al. (2006). High phenotypic dispersion in the data of studied traits will enhance the selection response in our planned for breeding program by selection index method.

Variance components

Table (2) show estimates of variance components, heritability (h^2) as well as genetic correlations (r_G) and phenotypic correlations (r_P) among different milk production and reproductive traits. The variance components of 305-dMY per kg, LP per day, Do per day and AFC per month are 281500, 273.1 , 134 and 7.471 for genetic variance, 1530000, 2438, 1276 and 26.21 for phenotypic variance, respectively. These estimates are in agreement with Hammoud (2013) working on a herd of Holstein cows in Egypt and reported that the variance components of 305-dMY per kg, LP per day and DO per day were 466296, 2848.64 and 3075.04 for genetic variance, 1102847, 5933.22 and 5741.82 for phenotypic variance, respectively.

Table (2) show estimates of heritability for 305-dMY, LP, DO and AFC are 0.184, 0.112, 0.105, and 0.285, respectively. These estimates are low to moderate and in agreement with most of the previous investigators. Heritability estimated were 0.17, 0.29 and 0.20 as reported by Meyer (1985), Dadpasand et al. (2013), Ghiasi et al. (2013) and Kaygisiz (2013) for 305-dMY; 0.06, 0.07 and 0.184 ±0.161 as reported by Lakshmi et al. (2009), El-Arian et al (2003) and Usman et al. (2012) for LP; 0.20 ±0.06, and 0.23 ±0.105 as reported by Salem et al (2006) for AFC.

Ghiasi et al. (2013) showed that heritability estimated of 305-dMY and DO were 0.32 and 0.076, respectively for Holstein cows. Endris et al. (2013) mention that estimated of heritability for 305-dMY of Holstein crossbred cows was 0.24 ±0.12, respectively.

In Egypt, heritability estimated of LP and DO were 0.38 and 0.42 (El-Shalmani 2011) and 0.04 and 0.20 (Shalaby et al. 2012) for first lactation of Friesian cows. Moreover, Hammoud (2013) showed that heritability estimates of 305-dMY, LP, and DO were 0.42, 0.48 and 0.54 for first lactation Holstein cows, respectively.

Osman et al. (2013b) showed that heritability estimates at the first parity of LP, DO and AFC were 0.107±0.07, 0.313±0.09 and 0.431±0.103, respectively for Holstein cows, respectively. Furthermore, the estimated LP and DO at the second parity were 0.166±0.077 and 0.117±0.071, respectively. Furthermore, Abdel-Gader et al. (2007) in Sudan and Tekerli & Kocak (2009) in Turkey found that heritability estimated of LP were 0.17 and 0.02 of Holstein cows, respectively.

The previous investigations revealed a substantial variation in heritability estimated AFC. High estimates were 0.48 and 0.42 as reported by Suhail et al. (2010) and Ayied et al. (2011), respectively. On the contrary, low heritability estimated of AFC was 0.098 as mentioned by Abdel-Gader et al. (2007).

Table 2. Heritability estimates (diagonal), genetic (below), phenotypic (above) correlation coefficients and variance components of studied traits for milk production and reproductive status in Holstein cows

Traits	305-dMY	LP	DO	AFC
305-dMY	0.184±0.032	0.037	-0.005	0.009
LP	0.406±0.131	0.112±0.025	0.894	0.092
DO	0.413±0.135	0.882±0.035	0.105±0.024	0.145*
AFC	-0.178±0.118	0.601±0.106	0.725±0.095	0.285±0.042
Listing of P-matrix				
305-dMY	1530000	2260	-220.9	57
LP	2260	2438	1577	23.26
DO	-220.9	1577	1276	26.52
AFC	57	23.26	26.52	26.21
Listing of G-matrix				
305-dMY	281500	3560	2536	-258.1
LP	3560	273.1	168.7	27.15
DO	2536	168.7	134	22.94
AFC	-258.1	27.15	22.94	7.471

Estimated genetic correlations (r_G) and phenotypic correlations (r_P) among previous traits were positive in general except between 305-dMY and DO (Table 2). Similar results were obtained by Ghiasi et al. (2013).

Selection index and the expected genetic gain

Heritability is used to calculate genetic evaluations, to predict response to selection, and to help breeders decide if it is more efficient to improve traits through management or through selection and making many practical decisions in breeding methods to predict the animal's estimated breeding value (EBV). By regarding heritability as the regression of breeding value on phenotypic value, an individual's EBV is simply calculated as the product of heritability and the phenotypic value. So, the moderate values of heritability for studied traits (305-dMY and AFC) will enhance the possibility of selection by raising its expected genetic gain per generation. König & Swalve (2009) revealed that correlations between indices and aggregate genotypes (r_{ti}) fall-down for traits with heritability's close to zero. There is a positive relationship between r_{ti} and heritability. Using Non-objective methods with modification in its charge to be negative for LP, DO and AFC to get higher desired genetic gain for traits under selection depends on the trait phenotypic dispersion.

General, reduced, sub as own-performance selection indices and multi-source of information are shown in Table 3. The general index is considered as the main index due to its properties, whereas this index contained all traits under selection program without any reduction. Furthermore, the general index is used as a standard efficient index to determine the relative efficiencies of the other types of selection indices.

Eighteen selection indices were constructed divided according to two strategies; first, strategy one include fifteen indices, and second, strategy two include three multi-source indices (Table 3).

The comparisons of the various selection indices indicated that the general index (I_1) which incorporated 305-dMY, LP, DO and AFC is the most efficient (R_{IH}=0.518; RE=100%) and it is recommended for improving milk Production and reproductive traits in Holstein cattle in Egypt in case of applying own-performance strategy. Similar results were obtained by Atil (2006) working on Friesian cow in Turkey, Using one standard deviation as a relative economic weight found that the general index incorporated 305-dMY, LP and AFC (I=.677*305-dMY+.06*LP-135.59*AFC) (R_{IH}=0.77) was the best and increase the expected genetic gain of 305-dMY by 346 kg/generation, LP increased by 3.37 day/ generation and AFC decreased by -1.62 mo/ generation.

Ghiasi et al. (2013) reported that the sub index which includes milk production trait (I=0.15*305-dMY) had the highest genetic gain for milk production (465 kg/generation), among the other selection indices.

However, the decline in fertility performance and profit was the opposite as observed in the index which had DO with 305-dMY (I=0.193*305-dMY - 1.7*DO) to get lower genetic gains for milk production (423 kg/generation). Similar results were reported by Gonzalez-Recio et al. (2006).

Missanjo et al. (2013) developed selection index which includes milk production and functional traits (I = 0.0004 milk yield + 0.0109 fat yield + 0.0313 protein yield + 1.0004 fat percent + 2.4491 protein percent − 0.1905 somatic cell count) and revealed that animals can be ranked according to this index and selection based on these rankings. The positive signs for production traits and negative sign for functionality trait mean that the index developed will allow breeders to select sires and dams, which will increas the production traits and decrease the functionality trait.

Ghiasi et al. (2015) reported that the fertility sub index which includes DO (I= 1.69*DO) had the highest correlated genetic gain for number of inseminations to conception (−0.25 time/ generation), and days from calving to first service (−8.6 day/generation). Therefore, this index had the highest profit per US dollar (3.5 US dollar /generation), among the other selection indices. Therefore, in conditions where fertility records are not available, DO can be used efficiently to improve fertility performance. These results suggest that two cows may have the same DO but different fertility performance either in the re-cycling activity post-calving or the ability to get pregnant. Further, censoring must be taken into account in genetic evaluations to improve predictive ability (González-Recio et al. 2006).

The least accuracy; first, in strategy one, [R_{IH} =0.17936 (I_9), 0.1793 (I_{13}), and 0.15827 (I_{14})] would result especially from indices that contain LP and DO in present study; second, in multi-source indices, [R_{IH} = 0.60475 (I_{18})] revealed the lower R_{IH} value in case of using maternal half-sibs as a second source of information. It is clear that the index not including AFC showed a reduction in its accuracy. Similar results were obtained by Khattab & Sultan (1991), Atil & Gevrekci (2005) and Atil (2006).

Table 3. Weighing factors (b-values), standard deviation (σi), efficiencies of selection in absolutes (R_{Ih}) and relative values (RE) in indices used to improve body weight at weaning in Holstein cattle

Selection index		Selection criterion				b-values				σi	R_{Ih}	RE (%)
						305-d MY	LP	DO	AFC			
GI												
I_1		MY	LP	DO	AFC	0.1267	-4.084	3.374	-52.77	326.47	0.518	100
RI												
I_2		MY	LP	DO	----	0.123	-3.039	0.985	----	188.85	0.300	57.85
I_3		MY	LP	----	AFC	0.123	-1.913	----	-51.27	322.09	0.511	98.66
I_4		MY	---	DO	AFC	0.120	----	-1.707	-51.24	313.92	0.498	96.15
I_5		----	LP	DO	AFC	----	-3.418	2.517	-52.22	286.78	0.455	87.84
I_6		MY	LP	----	----	0.122	-2.401	----	----	188.19	0.299	57.65
I_7		MY	---	DO	----	0.118	----	-2.772	----	176.58	0.280	54.09
I_8		MY	---	----	AFC	0.120	----	----	-52.96	308.07	0.489	94.36
I_9		----	LP	DO	----	----	-2.403	0.178	----	113.03	0.179	34.62
I_{10}		----	LP	---	AFC	----	-1.801	----	-51.1	283.99	0.451	86.99
I_{11}		---	---	DO	AFC	----	----	-1.733	-50.95	276.7	0.439	84.76
SI												
I_{12}		MY	---	----	-----	0.118	----	----	----	146.21	0.232	44.79
I_{13}		----	LP	----	-----	----	-2.288	----	----	112.99	0.179	34.61
I_{14}		----	---	DO	-----	----	----	-2.792	----	99.741	0.158	30.55
I_{15}		----	---	----	AFC	----	----	----	-52.7	269.84	0.428	82.65
MS												
	OP*	MY	LP	DO	AFC	0.076	-2.484	1.955	-30.01			
I_{16}	FS**	MY	LP	DO	AFC	0.426	-16.15	11.26	-78.46	466.55	0.740	142.91
	HS***	MY	LP	DO	AFC	0.105	-3.72	3.124	-33.98			
I_{17}	OP	MY	LP	DO	AFC	0.084	-2.69	2.113	-33.84	447.51	0.710	137.08
	FS	MY	LP	DO	AFC	0.461	-17.41	12.52	-91.14			
I_{18}	OP	MY	LP	DO	AFC	0.110	-3.615	3.019	-44.18	381.11	0.605	116.74
	HS	MY	LP	DO	AFC	0.151	-5.417	4.878	-50.49			

OP*= Own performance; FS**= Full sibs; HS***= Half sibs; GI= General index; RI= Reduced index; SI= Sub index; MS= Multi-source

Table 4. Expected genetic changes (eg) per generation and value of each trait in the index (vt) when using indices to improve body weight at weaning (*i = 1.0) in Holstein cows

Selection index		Selection criterion				Expected genetic changes (EG)				Value of each trait in the index (Vt %)			
						305-d MY (Kg)	LP (Day)	DO (Day)	AFC (Mo.)	305-d MY	LP	DO	AFC
GI													
I_1		MY	LP	DO	AFC	132.6	-4.679	-3.449	-1.410	12.16	3.84	1.34	42.15
RI													
I_2		MY	LP	DO	-----	139.1	-1.199	-0.367	-0.485	40.15	6.50	0.35	-----
I_3		MY	LP	-----	AFC	127.4	-4.585	-3.685	-1.449	11.83	4.35	-----	41.57
I_4		MY	-----	DO	AFC	135.8	-3.989	-3.504	-1.443	11.86	-----	1.86	43.75
I_5		-----	LP	DO	AFC	26.84	-6.717	-5.011	-1.483	-----	3.51	0.97	60.59
I_6		MY	LP	-----	-----	136.7	-1.181	-0.512	-0.513	39.96	22.31	-----	-----
I_7		MY	-----	DO	-----	148.0	-0.273	-0.411	-0.532	43.52	-----	17.2	-----
I_8		MY	-----	-----	AFC	154.2	-3.278	-2.954	-1.385	12.41	-----	-----	52.54
I_9		-----	LP	DO	-----	-71.7	-5.541	-3.376	-0.541	-----	11.76	0.03	-----
I_{10}		-----	LP	-----	AFC	23.88	-6.617	-5.197	-1.517	-----	4.99	-----	60.21
I_{11}		-----	-----	DO	AFC	31.64	-6.055	-5.063	-1.519	-----	-----	2.48	63.95
SI													
I_{12}		MY	-----	-----	-----	227.6	2.878	2.051	-0.209	-----	-----	-----	-----
I_{13}		-----	LP	-----	-----	-72.09	-5.531	-3.417	-0.550	-----	-----	-----	-----
I_{14}		-----	-----	DO	-----	-71.00	-4.723	-3.751	-0.642	-----	-----	-----	-----
I_{15}		-----	-----	-----	AFC	50.42	-5.302	-4.48	-1.459	-----	-----	-----	-----
MS													
I_{16}	OP*	MY	LP	DO	AFC	209.0	-6.37	-4.244	-1.843	1.85	0.65	0.21	4.65
	FS**	MY	LP	DO	AFC					5.24	2.32	0.47	3.11
	HS***	MY	LP	DO	AFC					1.15	0.44	0.16	2.10
I_{17}	OP	MY	LP	DO	AFC	200.1	-6.121	-4.082	-1.769	2.47	0.84	0.27	6.65
	FS	MY	LP	DO	AFC					6.91	3.03	0.65	4.72
I_{18}	OP	MY	LP	DO	AFC	161.1	-5.331	-3.827	-1.608	6.29	2.16	0.78	17.72
	HS	MY	LP	DO	AFC					3.72	1.44	0.59	7.47

OP*= Own performance; FS**= Full sibs; HS***= Half sibs; GI= General index; RI= Reduced index; SI= Sub index; MS= Multi-source; $*^i$ = Selection intensity

A positive relationship was found between 305-dMY and AFC (Table 2). It is necessary to select against the increase of LP more than 305 day, DO more than 60 days after calving and AFC more than 28 month of age as breeder's targets.

Strategy two includes four indices; the best restricted indices were I_{18}, I_{17}. It is suggested using I_{18}, I_{17} to improve milk Production and reproductive traits in Holstein cattle under restriction strategy. In case of populations that have already reached optimal 305-dMY, we suggest using completely restriction index (I_{19}) to get zero genetic gain in 305-dMY.

The original selection index (I_1) which included 305-dMY, LP, DO and AFC was suggested to be used for improving milk Production and reproductive traits in case of own-performance strategy.

The expected genetic change per generation (EG) in each trait assuming the selection intensity of 1.00 is given in table (4). The expected genetic change per generation (EG); first, in strategy one, ranged between -71.7 to 136.7 kg for 305-dMY, -0.2733 to -6.717 day for LP, -0.4109 to -5.197 day for DO and -0.2087 to -1.519 month for AFC; second, in multi-source indices, ranged between 161.1 to 209 kg for 305-dMY, -5.331 to -6.37 day for LP, -3.827 to -4.244 day for DO and -1.608 to -1.843 month for AFC.

The expected genetic gain after one generation through the general index (I_1) will be (1) increase in 305-dMY by 132.6 kg, (2) decrease in LP by -4.679 day, (3) decrease in DO by -3.449 day, (4) decrease in AFC by -1.41 month. This index is very simple and easy to construct, therefore, its use is recommended for selection for milk Production and reproductive traits in Holstein cattle in case of applying own-performance strategy.

The expected genetic gain after one generation through the full multi-source index (I_{16}) will be (1) increase in 305-dMY by 209 kg, (2) decrease in LP by -6.37 day, (3) decrease in DO by -4.244 day, (4) decrease in AFC by -1.843 month. This index is very useful to magnify the expected genetic gain, therefore, its use is recommended for selection for milk

production and reproductive traits in Holstein cattle in case of applying multi-source strategy.

Value of each trait in the index (Vt) as a percentage were illustrated in table (4) The Value of each trait in the index; first, in strategy one, ranged between 11.83 to 43.52 % for 305-dMY, 3.51 to 22.31 % for LP, 0.03 to 2.48 % for DO and 42.15 to 63.95 % for AFC; second, in multi-source indices, ranged between 1.15 to 6.91 % for 305-dMY, 0.44 to 3.03 % for LP, -0.21 to 0.78 % for DO and 2.10 to 17.72 % for AFC. These results reveal the importance of 305-dMY and AFC in the index because of the higher values of each trait in the index for these traits.

Atil (2006) working on Friesian cow in Turkey, Using one standard deviation as a relative economic weight reported that the expected genetic change per generation ranged from 321 to 402 kg for 305-dMY, 3.37 to 10.29 d for LP and 0.62 to -1.62 month for AFC. These results were lower than those reported by Atil & Gevrekci (2005) using another set of that herd and used actual economic values for and ranged from 363 to 411 kg for 305-dMY, 16.78 to 29.92 d for LP and from -0.35 to -0.65 mo for AFC. Also in this respect Khattab & Sultan (1991) working on Friesian cow in EGYPT, Using actual economic values found that the expected genetic gain per generation ranged from 88 to 235 kg for 305-dMY, from 21 to 27 d for LP and from -0.26 to – 1.96 month for AFC.

CONCLUSION

Results of this study suggested using the higher indexes of Rih ($I_{1\ (RE=100)}$) to improve milk production and reproductive traits in Holstein cattle under own-performance strategy and using ($I_{16\ (RE=142.91)}$) under multi-source strategy to get high accuracy and higher expected genetic changes per generation compare to general index.

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Heritability Estimation and Non-Genetic Factors Affecting Production Traits of Indonesian Ongole Cross

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ABSTRAK

Hartati, Muladno, Jakaria, Priyanto R, Gunawan A, Aryogi, Talib C. 2015. Estimasi nilai heritabilitas dan faktor non-genetik yang mempengaruhi sifat-sifat produksi sapi PO. JITV 20(3): 168-174. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1183

Data produktivitas dari 560 ekor sapi PO telah dikoleksi selama 9 tahun pengamatan dari 2004 sampai 2013 untuk mengkaji estimasi nilai heritabilitas dan pengaruh non-genetik terhadap sifat produksi sapi PO dari lahir sampai umur satu tahun. Estimasi nilai heritabilitas dilakukan dengan analisis variansi menggunakan data saudara tiri sebapak (*Paternal Halfsib Correlation*). Pemisahan komponen ragam untuk menduga nilai heritabilitas dilakukan dengan analisis sidik ragam Rancangan Acak Lengkap pola searah. Sedangkan pengaruh non-genetik (jenis kelamin, tahun, paritas, musim, generasi dan tipe lahir) dianalisis menggunakan *Generalized Linier Model* (GLM). Hasil penelitian menunjukkan bahwa jenis kelamin dan tahun berpengaruh nyata (P<0,05) pada sifat-sifat produksi yang meliputi bobot lahir, bobot sapih dan bobot setahun, sedangkan tipe lahir hanya berpengaruh nyata pada bobot lahir saja. Paritas, musim dan generasi menunjukkan pengaruh yang tidak nyata terhadap ketiga variabel produksi tersebut. Estimasi nilai heritabilitas bobot lahir, bobot sapih dan bobot setahun berturut-turut 0,28±0,12; 0,47±0,15 dan 0,63±0,17. Nilai heritabilitas tertinggi diperoleh pada bobot setahun, hal ini berarti bahwa bobot setahun efektif untuk digunakan sebagai kriteria seleksi dalam meningkatkan perbaikan genetik sapi PO.

Kata Kunci: Genetik, Non-Genetik, Produksi, Sapi PO

ABSTRACT

Hartati, Muladno, Jakaria, Priyanto R, Gunawan A, Aryogi, Talib C. 2015. Heritability estimation and non-genetic factors affecting production traits of Indonesian Ongole cross. JITV 20(3): 168-174. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1183

Productivity data from 560 head of PO cattle have been collected for 9 years from 2004 until 2013 for evaluating heritability estimation and non-genetic factors affecting production traits of Indonesian Ongole cross from birth to yearling old. Heritability estimation were analyzed using varians analysis with data of paternal halfsib correlation. Varians component for to estimate of heritability value were analyzed using completely randomized design one - way classification. While a general linear model was used to analyze non-genetic factors (sex, year, parity, season, generation and type of births). The results showed that sex of calves and year of births had significant differences (P<0.05) for all production traits such as weight at birth, weaning and yearling, while the type of birth only presented a significant difference on birth weight. Parity, season and generation exhibited nonsignificantly effect on those production traits. Estimation of heritability on birth weight, weaning weight and yearling weight were 0.28±0.12, 0.47±0.15 and 0.63±0.17 respectively. The highest heritability values obtained in yearling weight, this means that yearling weight will be effective as selection criteria to improve the genetic of PO cattle.

Key Words: Genetic, Non-Genetic, Production, PO Cattle

INTRODUCTION

PO cattle is one of the local cattle which is formed from the cross between Ongole sires on the island of Sumba (SO) with dam in Java that occurred decades ago (KepMenTan 2012). PO cattle is known to have a high diversity (Hartati et al. 2010), also have high adaptability and able to survive in conditions of tropical regions (Astuti 2004). These facts indicate performance of good productivity as good as other local cattle.

However, nowadays the existence of PO cattle has been widely reported to be degraded and decreased in genetic quality. Since the introduction of technology Artificial Insemination (AI) in the era of the 70s, almost 95% PO cows in farmer have been crossed with *Bos taurus* breeds. This crossing was initially thought to improve the productivity of local cattle, began to lose direction and purpose. Mostly the result of crosses actually used as broodstock and backcross to *Bos taurus* again, to increase *Bos taurus* genetic composition, it affected adaptability and decrease productivity as well

as contamination of the genetic quality of PO cattle. Currently, it is difficult to find PO cattle that have a good performance, even decline in productivity not only in weight and dimension of body size but also visible to reproduction aspects.

Various efforts have been done to improve the productivity of PO cow, one of which is through the selection program to produce quality cattle. Selection in principle is choosing animals that are genetically good quality to be used as a parent in the next generation, but in the implementation of this program is not provide optimal results. Selection conventionally takes a long time and a large population so that the selection program could only be applied to breeding livestock of government such as the Beef Cattle Research Station that does have the duties and functions to produce quality cattle, whereas in farmer which has the most population of PO cattle, selection programme can not be applied optimally because it is not supported by the data of production so the genetic potential are difficult to evaluate.

Beef Cattle Research Station as one of the technical management unit of Indonesian Agency for agricultural Research and Development Ministry of Agriculture has conducted breeding of PO cattle since 2004 until now, but the evaluation of genetic potential has not been done. Several result of studies have been reported, according to Adinata (2013) breeding value estimation for birth weight of PO cattle in Management Unit of Seed Source, heritability and repeatability were 0.686±0.525 and 0.805±0.041, whereas Prihandini et al. (2012) reported sire selection based on breeding values for weight 205 days and 365 days.

Evaluation of the genetic potential of livestock can be measured based on production and reproduction ability. The quantitative data of biological potency like production and reproduction phenotipic was inseparable from environmental influences where livestock were kept (Astuti 2004). Therefore, in addition to study about the effect of genetic such as heritability and breeding value estimation, the study of non-genetic influence was also done to eliminate biases caused by them such as the effect of sex, year, season, parity, generation and the type of birth. This study aimed to evaluate heritability estimation and non-genetic factors affecting production traits of PO cattle.

MATERIALS AND METHODS

Sources of data

In this study data used was records of PO cattle at Beef Cattle Research Station in Grati, Pasuruan, East Java for 9 years from 2004 to 2013. Productivity data from 560 head of PO calves have been collected from 253 head dams and 53 head sires. Data of individual animals that were available include calf, dam and sire number, date of birth, sex, parity, generation, type of birth, season and year of birth, weight at birth, weaning and yearling. Birth weight as the weight of animal in 24 hour after birth, weaning weight and yearling weight were obtained by calculating weight of animals from recorded data with the weight closest to the age of 205 days and 365 days respectively. Formulas used for calculating were as follows:

$$WW\ (205) = \frac{(AWW - B)}{AA} \times 205 + BW$$

$$YW\ (365) = \frac{(AYw - Weight\ 205)}{AA - 205} \times 160 + Weight\ 205$$

where:
AWW = Actual weaning weight
BW = Birth weight
AYW = Actual yearling weight
AA = Actual age

Feed ingredients used refers to the concept of Low External Input Sustainable Agriculture (LEISA) that utilize agricultural wastes were available throughout the season such as rice straw dry had ad libitum (+ 600 kg/7 days/20 head), concentrate feed 8 kg/head/day which were mix of tumpi, oil palm, rind coffee, bran quality 2, limestone and salt. Nutrien content of feed is presented in Table 1.

Table 1. The nutritional content of feed PO cattle appropriate physiological status

Physiological status	Nutritional content (DM)		
	CP (%)	TDN (%)	CF (%)
Dry periode	8-9	55-57	20-22
Mating, pregnant - weaning	9-10	57-60	18-20
Weaning calves - 24 month	9-10	58-60	20-22
Sire candidate (>18 month) and sire	10-12	58-60	20-22

Data analyses

Heritability value

Heritability estimation was calculated by analysis of variance using paternal halfsib correlations, where each bull mated with some cow and each cow has one progeny. Separation of variance components to estimate the heritability performed by analysis of variance Completely Randomized Design One-Way Classification with a mathematical model as follows (Becker 1992; Hardjosubroto 1994):

$$Yik = \mu + \alpha i + eik$$

where:
μ = Common mean
αi = Effect of the i□ʰ sire
eik = Uncontrolled environmental deviations associated with each record

Heritability value is estimated from sire variance components, according to Becker (1992) as follows:

$$h^2 = 4\ \delta^2 s / (\delta^2 s + \delta^2 w)$$

$$SE = 4\sqrt{\frac{2(1-t)^2\,[1+(k-1)(t)]^2}{k(k-1)(s-1)}}$$

where:
h^2 = Heritability
$\delta^2 s$ = Sire variance component
$\delta^2 w$= Within progeny variance component
k = Number of progeny per sire
s = Number of sires
$t = \delta^2 s / (\delta^2 s + \delta^2 w)$

Non-genetic influences

Data were analyzed to evaluate the effect of sex, year, season, parity, generation, and the type of birth on body weight at those certain ages. Seasons of birth in the year were divided into two seasons, namely dry (April-September) and rainy season (October to March). To determine the influence of non-genetic factors, data were analyzed using General Linear Model (GLM) (Steel & Torrie 1980) with the mathematical model as follow:

$$Y_{ijklmn} = \mu + r_i + s_j + p_k + q_l + n_m + m_n + e_{ijklmn}$$

where:
Y_{ijklmn} = Weight at birth, weaning, yearling and 2 years
μ = Overall mean
r_i = the effect of i^{th} years at birth (year 2003 to 2013)
s_j = the effect of j^{th} sex of calves (male, female)
p_k = the effect of k^{th}parities (1 to 8)
q_l = the effect of l^{th}seasons (dry, rainy)
n_m = the effect of m^{th}generations (1,2)
m_n = the effect of n^{th}birth types(1,2)
e_{ijklmn} = Random error

RESULTS AND DISCUSSION

Performance production of PO cattle

Statistical analysis of environmental influences on body weight of PO cattle and heritability estimation of birth weight, weaning weight and yearling weight are presented in Table 2 and 3.

Birth weight

Statistical analysis showed that sex, year and birth type significantly affected ($P<0.05$) birth weight in PO cattle, whereas parity, season and generation showed no significant effect. Birth weight of male calves was higher than that of the females 25.3 + 3.4 and 23.9 + 3.2 respectively (Table 2), whereas the overall average of birth weight of PO cattle was 24.6 + 3.4 kg. Birth weight in this research was lower compared to the results of Adinata (2013) who obtain birth weight PO cattle in Seed Resources Management Unit (UPBS) of 25.93±3.97 kg, but higher than the result reported by Supartini et al. (2014) birth weight PO cattle aged 4 days at the farmer of Tambakrejo subdistrict, Bojonegoro amounted to 11.07 + 3.92kg.

Effect of year on birth weight in this study was greatly fluctuate from year to year with the trend in 2010> 2013> 2004 (Table 2). The highest birth weight was obtained in 2013 with the average weight of 25.8 + 3.3 kg, whereas the lowest birth weight obtained in 2004 with an average of 22.3 + 3.0 kg, but in general the birth weight of 2004 to 2013 showed an increasing trend.

Birth type affected significantly ($P<0.05$) on birth weight, where calves form single birth had greater birth weight than those from twin birth. Difference of individual birth weight between twin and single birth is up to 7 kg or approximately 28.3%. Field observation and recording data analysis for 9 years in Beef Cattle Research Station shows that the probability of twinning in PO cattle around 1.4%. This result was in line with Komisarek & Dorynek (2002) that natural percentage of twin birth in beef cattle was less than 1%. Previous study reported that twinning birth in dairy cattle were between 1% and 4%. The probability of twinning could increase in line with the increasing of dam age where the rate of multiple births increased during the period of the 10 year age of the dams and and the greatest rise of

Table 2. Mean along with their standard deviation (SD) for birth, weaning and yearling weight (kg)

Performans of calves	Production traits		
	Birth weight (n)	Weaning weight (n)	Yearling weight (n)
Sex			
Male	25.3±3.4 (267)[a*)]	108.5±28.6 (264)[a]	157.1±40.6 (255)[a]
Female	23.9±3.2 (293)[b]	100.9±24.7 (291)[b]	147.3±38.1 (284)[b]
Years			
2004	22.3±3.0 (29)[a]	92.9±15.9 (29)[a]	119.7±25.0 (29)[a]
2005	22.8±3.4 (32)[a]	96.4±21.5 (32)[a]	135.6±25.4 (32)[a]
2006	24.6±4.3 (23)[a]	77.6±21.6 (23)[a]	111.9±34.8 (23)[a]
2008	25.6±3.4 (44)[ab]	106.6±27.1 (43)[ab]	147.7±38.8 (42)[ab]
2009	25.4±3.1 (71)[a b]	97.5±19.4 (71)[ab]	152.4±32.0 (71)[ab]
2010	24.0±3.2 (100)[a]	108.4±26.6 (100)[ab]	158.8±37.4 (99)[b]
2011	24.3±3.7 (65)[a]	94.3±20.7 (64)[b]	140.8±29.7 (63)[ab]
2012	24.3±2.8 (98)[a]	100.6±25.8 (98)[ab]	150.6±33.7 (98)[ab]
2013	25.8±3.3 (98)[b]	127.9±25.5 (96)[b]	183.8±45.8 (83)[c]
Parity			
1	23.9±3.4 (226)[a]	99.7±23.8 (226)[a]	146.0±34.9 (220)[a]
2	24.9±3.1 (142)[a]	105.2±29.7 (140)[a]	153.3±43.1 (135)[a]
3	25.1±3.6 (90)[a]	106.5±28.1 (89)[a]	150.6±43.4 (87)[a]
4	25.1±3.3 (57)[a]	115.2±27.3 (56)[a]	169.7±42.4 (54)[a]
5	24.7±3.7 (29)[a]	111.8±24.1 (29)[a]	166.3±32.0 (29)[a]
6	26.1±2.7 (16)[a]	104.6±27.1 (15)[a]	142.3±26.6 (14)[a]
Seasons			
Rainy	24.5±3.0 (195)[a]	105.4±27.1 (192)[a]	152.3±39.4 (179)[a]
Dry	24.6±3.6 (365)[a]	104.0±26.8 (363)[a]	151.8±39.7 (360)[a]
Generation			
1	24.7±3.5 (393)[a]	103.5±26.3 (389)[a]	149.7±39.1 (380)[a]
2	24.4±3.1 (167)[a]	106.8±28.2 (166)[a]	157.2±40.2 (159)[a]
Birth type			
1	24.7±3.3 (552)[a]	104.7±26.8 (547)[a]	152.2±39.4 (531)[a]
2	17.7±4.1 (8)[b]	88.6±31.1 (8)[a]	135.3±47.8 (8)[a]
Total average	24.6±3.4 (560)	104.5±26.9 (555)	151.9±39.6 (539)

*) Different of letters in the same section within column shows significantly different (P<0.05)

Table 3. Heritability estimation of body weight of PO cattle

Traits	n	h^2 ± SE	Vs	Ve	Vp
Birth weight	560	0.28±0.12	3.148	10.6	11.387
Weaning weight	555	0.47±0.15	338	640	724.50
Yearling weight	539	0.63±0.17	997.47	1325	1574.367

n= Number of calves; h^2 = Heritability; Vs= Sire variance; Ve= Environment variance; Vp= Phenotypic variance

twinning occurred in first and second parity (Berry et al. 1994; Cady & Van Vleck 1978; Kinsel et al. 1998; Nielen et al. 1989; Ryan & Boland 1991), whereas in this study twinning occured in first, third and fifth parity.

Result of heritability estimation for birth weight of PO cattle was 0.28 + 0.12 (Table 3). The heritability in this study was lower than heritability of other local cattle. Gunawan et al. (2011) reported heritability of birth weight for Bali cattle amounted 0.33 + 0.99. Whereas Adinata (2013) reported heritability of birth weight for PO cattle in UPBS groups was higher namely 0.686±0.525. Heritability of birth weight in this study was similar to heritability of birth weight for Zebu breed reported by Albuquerque & Meyer (2001) 0.28. Boligon et al. (2009) reported that heritability for birth weight of Nellore cattle (*Bos indicus*) was 0.25. Araujo et al. (2014) reported estimation of heritability for birth weight of Nellore Cattle in The Midwest region of Brazil was 0.37±0.02. This is very relevant considering that PO and Nellore cattle are two breeds that have a kinship. Historically, Nellore cattle was Ongole breed imported from India and developed in Brazil and PO cattle was a crossbred between bull of Ongole from India in the Sumba island (namely SO) with Java cows. Generally, the value of heritability on birth weight in this study was quite moderate and within the range of published values by Hardjosubroto (1994) 0.2-0.58, these result show that the correlation of phenotypic variances and genetic variances is quite moderate so the selection based on the phenotypic of the individual birth weight to be quite effective. Lasley (1978) stated that selection on birth weight can be done to improve genetic quality weaning weight trait, yearling and weaning daily gain due to the high correlation of genetic variances in these traits.

Weaning weight

The overall average of weaning weight of PO cattle was 104.5±26.9 kg. Weaning weight in this research was lower than that reported by Aryogi et al. (2006) that weaning weight of PO cattle at the farmer in East Java was 109 kg. Statistical analysis of the environmental influence showed that sex and year were significant (P<0.05) on weaning weight of PO cattle (Table 2). Maylinda (2010) stated that weaning weight was influenced by many environmental factors, especially the management and maintenance of the genetic component of the parental (*maternal genetic effects*) such as the production of milk and breast feeding behavior that ultimately affected the performance of individuals. It is very relevant to this study, in which the male calves have greater (108.5±28.6 kg) weight than the females (100.9±24.7 kg). This is likely due to male calves have a greater ability to stimulate the production of mother's milk during suckling, so that the male consumes more nutrients than the female. Thus weaning weight can also be used as a criterion in selecting the dam that has good mothering abilities.

Effect of year on weaning weight in this study was greatly fluctuate from year to year with the trend in 2010> 2013> 2006 (Table 2). The highest weaning weight was obtained in 2013 with average of 127.9±25.5 kg, whereas the lowest weaning weight was obtained in 2006 an average of 77.6±21.6 kg.

Heritability estimation for weaning weight of PO cattle was 0.47±0.15 (Table 3). Heritability value in this study was higher than heritability commonly found in offspring of Ongole breed in some other countries. Silva et al. (2013) reported heritability of Ongole breed developed in Brazil or known as Nellore cattle was 0.13. While Araujo et al. (2014) found heritability was 0.36. Heritability can varies higher or lower from one study to other studies, it is likely influenced by the ability of the environment that will reduce the influence of pure genetic elements in cattle. But, generally heritability in this study was high category due more than 0.4 (Noor 2010). The high value of heritability of weaning weight showed that the selection on the basis of performance of the individual will be more effective to increase daily gain in weaning weight that indicates these traits can increase the response selection.

Yearling weight

The overall average of yearling weight of PO cattle was 151.9 + 39.6 (Table 2), lower than yearling weight the result reported by Aryogi et al. (2006) 166.4 kg. Statistical analysis of the environmental influences showed that sex and year were significant (P<0.05) on yearling weight of PO cattle (Table 2). Result showed that the average of yearling weight of male PO cattle was higher than female: 157.1 + 40.6 dan 147.3 + 38.1, respectively. The difference of this weight might be attributed to different physiological processes in both sexes. Dadi et al. (2008) stated that sex had a highly significant influence on post weaning live weight and growth rate in Brahman cattle, differences in growth rate increased with age implying that sex effects are more pronounced with age after puberty. The result of this study describing that the influence of sex on live weight difference increased with age from 7.6 kg at weaning weight of age to 9,8 kg at yearling weight.

Effect of year on yearling weight in this study greatly fluctuate from year to year (Table 2). The highest yearling weight was obtained in 2013 with average of 183.8±45.8 kg, whereas the lowest yearling weight was obtained in 2006 with an average of 111.9±34.8 kg, but in general the yearling weight from 2004 to 2013 showed an increasing trend.

Heritability values have been calculated for direct genetic effect of yearling weight was 0.63 + 0.17 (Table 3). These results were higher than some of the Ongole breed heritability values previously reported in the literature. Silva et al. (2013) reported a heritability estimation of yearling weight of Nellore cattle using a mixed model of analysis of 0.03 + 0.02. While Araujo et al. (2014) reported the estimated heritability of Nellore cattle in the Midwest region of Brazil 0.31 + 0.01. Santos et al. (2012) also reported a heritability values of 0.51 for yearling weight in which these values are lower than result of this study. The heritability value differences might be caused differences in the number of data structures, management and genetic data analysis methods. Clement et al. (2001) suggest that genetic variance was influenced by differences in the data number (structure) analyzed, the method of genetic analysis, the relationship among cattle herds and research time. The results of this study was an indication of good genetic progress for the growth traits of zebu cattle, especially in the tropical regions. Direct heritability estimation for body weight at evaluation of different age have medium and high value, it was indicating that additive genetic diversity sufficient to give response to selection. This result showed an indication that yearling weight can be used as an effective criterion for selection to improve growth rate in PO cattle.

CONCLUSION

Environment has significant influence (for sex and year) on: birth, weaning and yearling weight. Male calves have higher body weight than female calves, whereas performance of body weight of PO cattle was greatly fluctuate from year to year. Heritability estimation on birth, weaning and yearling weight were 0.28 + 0.12; 0.47 + 0.15 and 0.63 + 0.17 respectively. Heritability estimation on body weight of PO cattle was considered medium and high category. The highest heritability values obtained in yearling weight, this means that yearling weight will be effective as selection criteria to improve the genetic of PO cattle.

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Antimicrobial and Antioxidative Activities of Peptides from Goat Milk Hydrolyzed with Various Protease

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ABSTRAK

Kusumaningtyas E, Widiastuti R, Kusumaningrum HD, Suhartono MT. 2015. Aktivitas antimikroba dan antioksidan peptida hasil hidrolisis susu kambing dengan berbagai protease. JITV 20(3): 175-183. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1184

Susu mempunyai nilai nutrisi tinggi dan mengandung protein sebagai sumber peptida bioaktif yang berguna bagi kesehatan. Penelitian ini bertujuan untuk mengeksplorasi potensi peptida bioaktif dari susu kambing sebagai antimikroba dan antioksidan. Susu dihidrolisis menggunakan enzim tripsin, kimotripsin, pepsin atau protease *Bacillus* sp. E.13. Peptida yang diperoleh dipilah untuk aktivitas antimikroba dengan mencampurkan peptida dan bakteri *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella thyphimurium* dan *Escherichia coli* sebanyak 10^6 CFU/mL dan diinkubasi pada suhu 37°C selama 2 jam dan ditumbuhkan pada *Mueller Hinton* agar. Aktivitas antimikroba ditentukan dengan membandingkan jumlah koloni bakteri yang tumbuh pada cawan dengan dengan jumlah koloni bakteri kontrol tanpa penambahan peptida. Aktivitas antioksidan ditentukan melalui uji 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) dan 2,2-diphenyl-1-picrylhydrazyl (DPPH). Aktivitas antimikroba terlihat pada peptida hasil hidrolisis susu kambing oleh pepsin pada suhu 37°C, pH 2 selama 90 menit dan protease *Bacillus* sp. E.13 pada suhu 55°C, pH 11 selama 30 and 60 menit tetapi aktivitas tersebut tidak terdeteksi pada peptida hasil hidrolisis protein dengan tripsin dan kimotripsin. Peptida dari hidrolisis protein oleh protease *Bacillus* sp. E.13 dapat menghambat *Listeria monocytogenes*, *Salmonella thyphimurium* dan *Escherichia coli* sampai 5 siklus log. Peptida antimikroba tersebut juga dapat meredam radikal ABTS sampai 86% dan radikal DPPH 9% pada konsentrasi 68 µg protein/mL. Hasil tersebut mengindikasikan bahwa protein susu kambing yang dihidrolisis dengan protease *Bacillus* sp. E.13 berpotensi sebagai antimikroba sekaligus sebagai antioksidan.

Kata Kunci: Susu Kambing, Peptida, Antimikroba, Antioksidan

ABSTRACT

Kusumaningtyas E, Widiastuti R, Kusumaningrum HD, Suhartono MT. 2015. Antimicrobial and antioxidative activities of peptides from goat milk hydrolyzed with various protease. JITV 20(3): 175-183. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1184

Milk is highly nutritious food containing protein as a good source of bioactive peptide that beneficial for health. This research was aimed to explore potency of bioactive peptide derived from goat milk as an antimicrobial and antioxidant. Milk was hydrolyzed by trypsin, chymotrypsin, pepsin, or protease *Bacillus* sp. E.13. The peptides obtained were screened for antimicrobial activities through incubation with *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella thyphimurium* and *Escherichia coli* at 10^6 CFU/mL at 37°C for two hours and plated on Mueller Hinton agar. Antimicrobial activities were determined by comparing the total bacterial colonies to that of bacterial control without peptides addition. Oxidative activity was determined by 2.2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 2.2-diphenyl-1-picrylhydrazyl (DPPH) assays. Antimicrobial activities were shown in peptides produced from hydrolysis of goat milk protein by pepsin at 37°C, pH 2 for 90 min and by *Bacillus* sp. E.13 protease at 55°C, pH 11 for 30 and 60 min but the activities were not detected in peptides from hydrolysis by trypsin and chymotrypsin. Peptide from protein hydrolysis by *Bacillus* sp. E.13 protease could inhibit *Listeria monocytogenes*, *Salmonella thyphimurium* and *Escherichia coli* up to 5 log cycles. The antimicrobial peptides could scavenge ABTS radical up to 86 % and DPPH radical up to 9 % at 68 µg protein/mL. Results indicated that goat milk protein hydrolyzed by *Bacillus* sp. E.13 protease is potential as antimicrobes and antioxidant.

Key Words: Goat Milk, Peptide, Antimicrobe, Antioxidant

INTRODUCTION

Milk is highly nutritious food characterized by its amino acid profile balance. Concentration and composition of milks produced by each mammal are different depending on physiology and structure needed by the mammal's infants (Potocnik et al. 2011) affecting its essential amino acid and bioactive peptides

contents. The bioactive peptides from cow milk have been reported could be antimicrobials, antioxidants, antihypertensives, and antithrombotics and some of them have multi functions (Lopez-Exposito et al. 2007). Amino acid sequences of proteins from cow and goat milks have the similarity which may express similarity in their bio-activities. Nevertheless, variation of amino acids from those species brings through variation in the bio-activaties.

Besides the amino acid sequence, the enzyme specificity used to hydrolyze protein was another factor for bioactive peptide produced (Pihlanto 2006). Bioactive peptides are mostly obtained through activity of indigenous protease or from enzyme intentionally added. Each enzyme has different substrate specificity and hydrolysis different amino acid sequence that produces different peptide fragments with certain amino acid sequence and bioactivity. Hydrolysis conditions such as pH, temperature, and hydrolysis time determine the length of peptide sequences those also affects their activities. In this study, the goat milk protein was hydrolyzed by enzymes, and then antimicrobial and antioxidant activities of peptides produced were determined.

Antioxidant is defined as a substance in a small concentration that inhibit substrate oxidation (Halliwell 1990). The cell oxydation may produce reactive oxygen species (ROS) those attack macromolecules such as membrane lipida, protein and DNA and cause the pathogenesis of hypertension and stroke (Greig et al. 2010). Excessive nitric oxide (NO) produced during inflammation process affect pathologic problems to animal or human cells (Kawanishi et al. 2006). Antioxidant has a role to decrease the negative effects.

Bioactive peptides from hydrolyzation of goat milk by human gastrointestinal enzyme produced antibacterial and antioxidant compounds known to inhibit the growth of *Escherichia coli* K12, *Bacillus* cereus RT INF01, *Listeria monocytogenes* and *Staphylococcus aureus* ATCC 25 923 (Almaas et al. 2011). For antioxidant activity, ability of scavenging free radical and chelating Fe ion were shown by peptide and casein of goat hydrolyzed milk using combination of neutral protease and alkali (Li et al. 2013).

Enzymatic hydrolysis is an efficient technique generally used to produce bioactive peptide from natural protein (Madureira et al. 2010). Digestive enzymes used in this study were trypsin, chymotrypsin, pepsin, and protease from *Bacillus* sp. E.13. The digestive enzymes have been used to hydrolyze cow milk protein to produce bioactive peptides (McCann et al. 2005). *Bacillus* sp. E.13 was high proteolytic bacterium isolated from Bogor horse milk. This study was aimed to explore the potency of goat milk produced the bioactive peptide as antimicrobial and antioxidant through enzymatic process.

MATERIALS AND METHODS

Microorganism

Bacillus sp. E.13 was used for production of protease, while microorganisms used for antibiotic assays were *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 15313), *Escherichia coli* (ATCC 25922) and *Salmonella Typhimurium* (ATCC 13311).

Goat milk

Milk of Etawa crossbread goat was obtained from Faculty of Animal Science, Bogor Agricultural University. Fresh milk fat was separated by centrifugation at 6000 g for 15 minutes and discarded, while whey and casein was remixed and hydrolyzed.

Determination of protein concentration

Determination of protein concentration was done using Bradford (Quick start TM Bradford protein assay, Bio-Rad Inc). Standard curve was made by reacting 5 µl of Bovine serum albumin (BSA) in various concentrations with 95 µl Bradford solution. The same treatment was done to samples and distilled water as a blank. The reactions were incubated at room temperature for 5 minutes and their absorbance were measured in λ= 600 nm (Labsystems, original Multiscan Ex).

Production of *Bacillus* sp. E. 13 protease

Bacillus sp. E.13 was grown in Luria bertani broth consisting of 0.05 % skimmed milk incubated at 37ºC for 24 hours. Culture was centrifuged at 3500 g for 15 minutes and enzyme in the supernatant was precipitated with 50% ammonium sulfate according to Rowan et al (1990). After overnight incubation at 4°C pellet of the enzyme was collected by centrifugation at 1000 g for 15 minutes. The pellet was wind-dried and stored at -20ºC before used or dissolved in PBS pH 7.4 with ratio 1:2 for direct use to hydrolyze milk protein.

Determination of enzyme activity

Protease activity determination of enzymes was assayed using the method of Bergmeyer & Grassel (1983) by reacting 250 µL 2% (w/v) casein with 50 µL enzyme and 250 µL PBS 0.05 M pH 7. The mixture was incubated at 37ºC for 10 minutes and then 500 µL TCA 0.2 M was added and incubated again at 37ºC for 10 minutes, then was centrifuged by 2000 g for 10 minutes. Supernatant was separated and 375 µL of the supernatant was mixed with 1250 µL Na_2CO_3 0.4 M

and added with 250 µL Folin Ciolcateau reagent with 1:2 dilution and incubated at 37ºC for 20 minutes. Absorption was measured at λ 578 nm. Distilled water was used as a blank and 5 mM tyrosine solution was used as standard. One unit of activity was defined as amount of enzyme which could produce 1 µmol tyrosine per minute at assay condition.

Hydrolysis of milk protein

Hydrolytic condition of trypsin (T1426, Sigma-Aldrich Co, 10.000 BAEE unit/mg protein), chymotrypsin (C4129, Sigma-Aldrich Co, 40 unit/mg protein) and pepsin (P7000, Sigma-Aldrich Co, 250 unit/mg solid) was carried out based on instruction of each enzyme namely: trypsin was at 37ºC, pH 8 for 120 minutes, in the ratio between enzyme and substrate of 1:100; chymotrypsin was at 30ºC, pH 7.8 for 120 minutes with enzyme and substrate ratio was 1:60; and pepsin was at 37ºC, pH 2 for 60 minutes with enzyme and substrate ratio was 1:30. For protease of *Bacillus* sp. E.13, enzyme activity was 0.67 unit/mL with enzyme and substrate ratio was 1:20. The hydrolysis was done at 55ºC and pH 7 (Josephine et al. 2012) and pH 11 (Patel et al. 2006) for 30-60 minutes. Each peptide from the hydrolysis was centrifuged at 14000 g for 15 minutes until 3 layers formed. Transparent-colored center was collected for antimicrobial and antioxidant tests.

Antimicrobial test

Antimicrobial test was done using *Staphylococcus aureus, Listeria monocytogenes, Salmonella thyphimurium* and *Escherichia coli*. Screening was done using screening method by Lopez-Exposito et al. (2007), that was by mixing 100 µL peptide with 100 µL microbes at 10^6 CFU/mL in microplates and incubated at 37ºC for 2 hours. Then, 10 µL of the mixture was dripped on Mueller Hinton agar and incubated at 37ºC overnight. The result was positive if there is a bacterial growth inhibition. Every treatment was repeated 3 times. The next test was done by mixing 100 µL peptide with 100 µL bacteria at 10^6 CFU/mL in microplates and incubated at 37ºC for 2 hours. Then the mixture was diluted and grown on Mueller Hinton agar and incubated again at 37ºC for 24 hours. Colonies of bacteria were counted.

Antioxidant activity assay using ABTS [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)]

Stock solution of 7.4 mM ABTS was prepared in deionized water, while stock solution of potassium persulfate 2.4 mM. Before assays the reactant was prepared by mixing both stock solutions by 1:1 ratio. Oxidation occurred when the mix solution kept in dark condition for 16-18 hours. The solution was diluted with deionized water to obtain absorbance by 1.1±0.02 unit at λ 405 nm. Peptides at 100 µl were mixed with 200 µl ABTS radical solution in microplates and incubated for 10 minutes. The absorbance was read at 405 nm. This method was carried out according to Thaipong et al (2006) with replacing cuvette with microplates. Standard curve was made by measuring antioxidant activity of vitamin C p.a at various concentrations. Determination of every sample activity was repeated 3 times.

Antioxidant activity assay using DPPH (2.2-diphenyl-1-picrylhydrazyl)

Antioxidant testing using DPPH was done based on modified combination methods of Thaipong et al. (2006) and Clarke et al. (2013). Ethanol 96% was used for diluting DPPH at absorbance 1.1±0.05 instead of methanol (Thaipong et al. 2006). The modification was carried out after preliminary test in both methanol and ethanol showed relatively similar. Peptides at 100 µl in various concentrations were added with 200 µl DPPH and left for 30 minutes and the absorbance was measured at λ= 540 nm (Clarke et al. 2013). Standard curve was made by measuring the antioxidant activity of vitamin C p.a in various concentrations. Every sample was determined in triplicate.

Antioxidant activity in ABTS and DPPH test was calculated by the following equation:

$$Antioxidant\ activity\ (\%) = \frac{(Blank\ abs - Sample\ abs)100\%}{Blank\ abs}$$

where:

Blank abs = Was an absorbance of ABTS/DPPH solution

Sample abs = Was absorbance of samples (pepti des) reacted with ABTS/DPPH minus abs of peptide as controls. The controls were prepared without ABTS/DPPH

Vitamin C equivalent was calculated based on standard curve of vitamin C antioxidant activity

Profile of peptides using HPLC

Selected peptide hydrolysates those had the best microbial activity incubated at 30 and 60 minutes were applied into HPLC C-18 column (5 µm, 4.6 x 250 mm, Xterra, Waters). Hydrolysates were eluted with gradient linear 5-45% (v/v) of 0.1 % trifuoroacetic acid (TFA) (v/v) in acetone nitrile (ACN) (solvent A) in 0.1% TFA (v/v) in the deionized water (solvent B). HPLC system was equilibrated by 95% of solvent B for 5 minutes, followed by the gradient solvent for 16 minutes to elute

peptides, then re equilibrated with solvent B for 5 minutes (McCann et al. 2005). Absorbance was measured at λ= 215 nm.

RESULTS AND DISCUSSION

Data from Table 1 showed that only peptides from goat milk hydrolyzed with proteases of *Bacillus* sp. E.13 at pH 11 and pepsin at 90 minutes incubation which inhibited bacterial growths. Filtered goat milk at 0.45 µm as a control also showed negative inhibition that confirmed that only hydrolyzed milk peptides had antimicrobial activity. Using other enzymes and hydrolysis conditions also showed negative results. Inhibition of pepsin as protease might be influenced by the low pH condition, since the pepsin itself also showed inhibition, while other enzymes in neutral and alkali condition gave negative results. Further research should be carried out to determine whether the antimicrobial activity was from peptide produced or due to low pH.

Peptides of goat milk hydrolyzed by trypsin and chymotrypsin did not inhibit the growth of any tested bacteria (Table 1). These data are in agreement by data reported by McCann et al. (2006) which using peptides of cow milk casein hydrolyzed by trypsin and chymotrypsin for 4 hours. Burris (2004) reported that cow milk casein hydrolyzed by trypsin and chymotrypsin in the same temperatures and pH hydrolysis as in this experiment but longer incubation time for five hours could inhibit the growth of *L. monocytogenes*. Addition of hydrolysis time as done by Burris (2004) might cause further hydrolysis producing shorter peptides and higher antimicrobial activity.

In this experiment hydrolysis time was carried out according to the enzyme label, longer incubation time that produce more active peptides will be good to be evaluated. There is a possibility that difference sequence of amino acids of cow milk used by Burris (2004) with goat milk in this study resulted different antimicrobial activity. Peptide from goat milk hydrolyzed by pepsin for 90 minutes in this experiment only inhibited 1 log cycle against *S. aureus, L. monocytogenes, E. coli* and *S. Typhimurium*.

Further antimicrobial activity determination using colony form unts showed that peptides of goat milk hydrolyzed by protease *Bacillus* sp. E.13 for 30, 60 minutes only decreased CFU of *S. aureus* less than 1 log cycle, while for *L. monocytogenes, E. coli* and *S. Typhimurium* could decrease up to 5 log cycles (Figure 2). Longer hydrolysis incubation time from 30 to 60 minutes did not affect CFU numbers.

Those results proved that protease from *Bacillus* sp. E.13 could potentially hydrolyze goat milk protein into antimicrobial peptides especially for *L. monocytogenes, E. coli* and *S. Typhimurium* which are Gram-positive and negative bacteria. Some *Bacillus* proteases are reported may be used to hydrolyze protein into antimicrobial peptides with high activity. Kent et al. (2012) reported that bovine casein fermented by *B. cereus* and *B. thuringiensis* inhibit *Chronobacter sakazakii*. Hydrolysis of goat milk casein using *Bacillus* sp. P45 produces antimicrobial peptides against *S. enteritidis, E. coli, C. fimi* and *L. monocytogenes* (Daroit et al. 2012).

Table 1. Antimicrobial activity of peptides from goat milk hydrolyzed with various enzymes, pH, temperature and incubation time conditions

Bacteria	Antimicrobial Activity							
	Trypsine	Chymotrypsine	Pepsin		*Bacillus sp*.E.13 Protease			
	37ºC	30ºC	37ºC		55ºC		55ºC	
	pH 8	pH 7.8	pH 2		pH 7		pH 11	
	120’	120’	60’	90’	30’	60’	30’	60’
S. aureus	-	-	-	+	-	-	+	+
L monocytogenes	-	-	-	+	-	-	+	+
E. coli	-	-	-	+	-	-	+	+
S. typhimurium	-	-	-	+	-	-	+	+

+: positive result for antimicrobial activity
- : negative result for antimicrobial activity

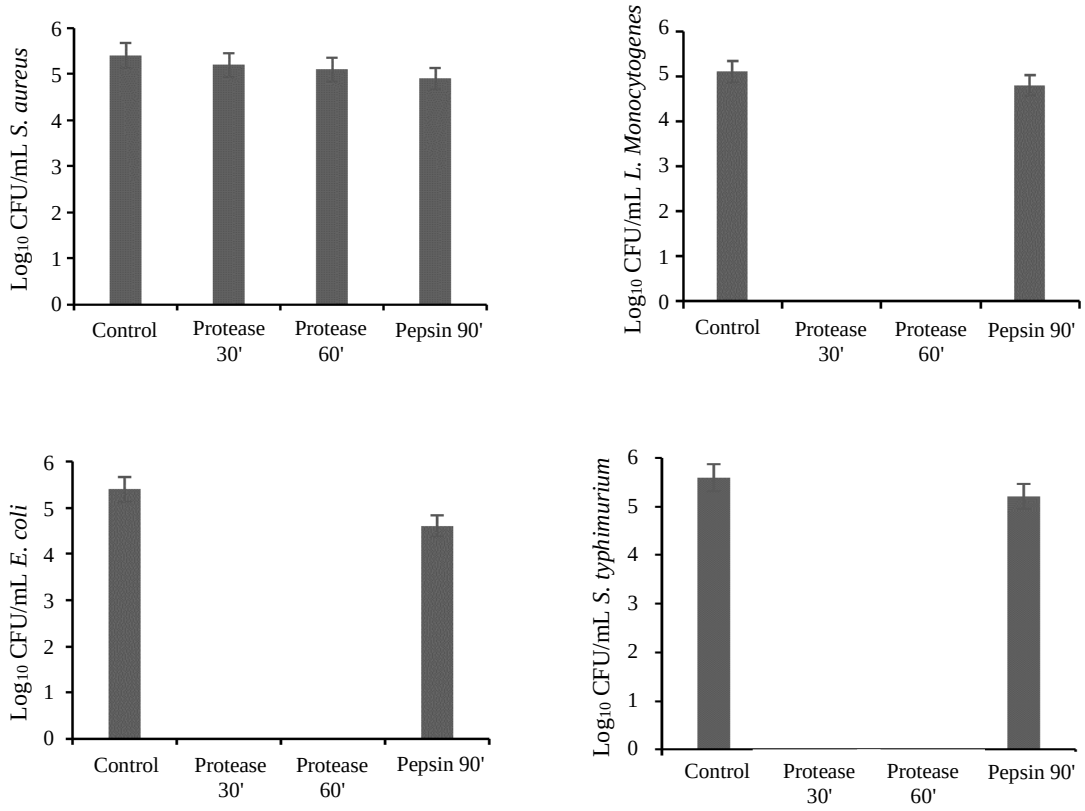

Figure 1. Number of CFU of bacteria from antimicrobial testes of milk goat peptides produced by hydrolysis with proteases of *Bacillus* sp. E.13 at 55ºC pH 11 for 30, 60 minutes and pepsin at 37ºC pH 2 for 90 minutes, respectively

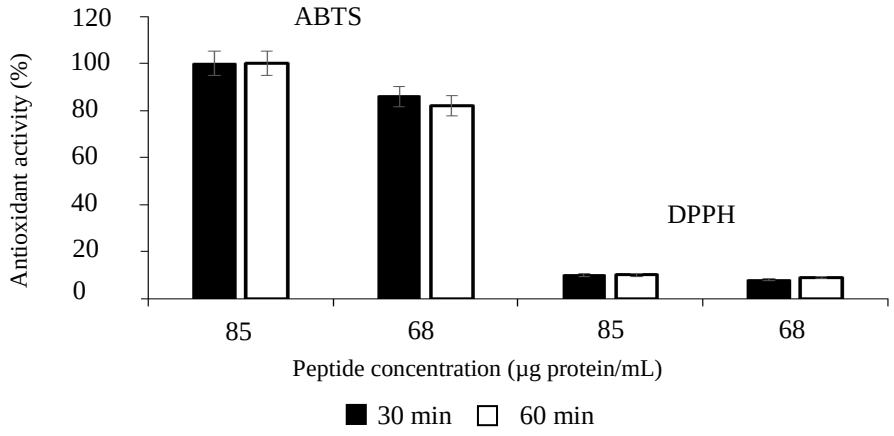

Figure 2. Antioxidant activities of peptides from goat milk hydrolyzed by protease *Bacillus* sp. E.13 at 55ºC pH 11 for 30 at 60 minutes at concentrations of 68 and 85 µg protein/mL against ABTS and DPPH

In this study, *S. aureus* was more resistant against antimicrobial peptide compared to other bacteria. There is possibility in this experiment that antimicrobial peptide formed pores in other bacteria cell membrane, broke the membrane and destroy the cells. Rigidity of cell wall in *S. aureus* persistents to turgor pressure up to 3-25 higher than that may be tolerated by the Gram-negative bacteria (Sato & Feix 2006). That ability led

the *S. aureus* relatively more resistant to antimicrobial activity. The bacterium is capable to produce an extracellular protease such as aureolysin which may breaks down the LL-37 antimicrobial peptide by hydrolyzing its C-terminal (Nawrocki et al. 2014).

Although *L. monocytogenes* is a gram-positive bacterium, its growth was inhibited by peptide from goat milk protein hydrolyzed by protease *Bacillus* sp. E.13 at 37°C. Lopez-Solanilla et al. (2003) reported various susceptibilities of *L. monocytogenes* to antimicrobial peptides and influenced by their incubation temperatures. *Its* growth was highly inhibited by human defensin but slightly inhibited by protamine peptide, snakin, and magainin. Defensin peptide of potato was very affected in inhibition of the bacterium at 37ºC, but at 20ºC the bacterium is resistant (Lopez-Solanilla et al. 2003).

Neutralization of ABTS and DPPH radicals was measured based on the ability of antioxidant to give hydrogen atom or to break down the radical compound (Correa et al. 2011). Antioxidant assays using ABTS is able to use in aqueous and organic solvent and not influenced by ionic strength. Therefore, the assay is applicable to determine hydrophilic or hydrophobic antioxidant (Prior et al. 2005). DPPH was usually used as ABTS for comparison, even though the ABTS usually gives higher antioxidant activity of various food materials (Floegel et al. 2011).

In ABTS assay, goat milk hydrolyzed by protease *Bacillus* sp. E.13 with protein concentration at 68 µg/mL from 250 times dilution had antioxidant activity of 83% for 30 minutes hydrolysis and 86% for 60 minutes hydrolysis. The peptide neutralized ABTS radical up to 100% at 200 times dilution. The peptide from 30 minutes hydrolysis had higher activity to neutralize the ABTS radical than that of peptide from 60 minutes hydrolysis (Figure 2). Longer time hydrolysis of goat milk protein using protease *Bacillus* sp. E.13 decreased antioxidant activity. Antioxidant activity against ABTS increases after the milk casein hydrolyzed by protease compared with the one before hydrolysis (Rossini et al. 2009). Hydrolysis releases peptides in smaller size, increases ionized group, and discloses hydrophobic group which influence antioxidant activity (Sarmadi & Ismail 2010). Correa et al. (2011) showed that the ability of casein hydrolyzed by protease of *Bacillus* sp. P7 to neutralize ABTS radical increasing by time incubation up to two hours, after that it becomes plateau.

Daroit et al. (2012) reported that antioxidant activity of peptide from goat milk casein hydrolyzed by protease *Bacillus* sp. P45 increased after 4 hours. That results was different with data from this experiment that decrease activity was observed in longer hydrolysis time from 30 up to 60 minutes. Sun et al. (2011) described that there was no correlation between hydrolysis time and antioxidant activity. Enzyme specificity on amino acid sequences to produce peptides more determine antioxidant activity than time of hydrolysis. Purification may increase antioxidant activity, if the process increase the concentration and purity of the active peptide. However, antioxidant activity may decrease if purification omits interaction between peptides in hydrolysate. Antioxidant activity was higher by mix peptides of HAHp1-2III (901.45 Da), HAHp1-2IV (872.37 Da and HAHp1-2V (1171.60 Da) from half-fin ancovy protein than the singles (Song et al. 2014).

In DPPH assay, detected antioxidant activity was lower than that of ABTS assay. The difference may due to difference of DPPH and ABTS character. DPPH radical is more stable than ABTS radical resulting DPPH more dificult to be neutralized (Prior et al. 2005). DPPH also has narrow of reaction range than ABTS, therefore it need more peptide to reach the same antioxidant activity value with ABTS. However, they have similar value of vitamin C equivalent. For example, antioxidant activity of peptide from 30 minutes hydrolysis with concentration 35 µg protein/mL equal with ±3 µg/mL of vitamin C to neutralize the ABTS radical and equal with ability of ±2.55 µg/mL vitamin C to neutralize the DPPH radical.

Characterization of peptides using RP-HPLC is shown in Figure 3. Longer hydrolysis time at 2.5 minutes retention time increased more peptide from goat milk hydrolyzed by protease *Bacillus* sp. E.13. Peak for 30 minutes hydrolysis increased from 0.6 AU into 1.35 AU at 60 minutes hydrolysis. On the contrary, the peptide with 8-10 minutes of retention time decreased intensity from 0.80 AU into 0.60 AU respectively indicating a decrease of peptide concentration with 8-10 minutes of retention time.

The absorbance of the peptide hydrolysates was conducted at 215 nm, the specific wavelength for peptides. Based on the chromatogram (Figure 3), character of the peptides from 30 and 60 hydrolysis are almost similar, indicating possibility of similar peptide composition. Change of peptide intencities may related to their bioactivities, but it need further anaysis such as amino acid sequencing using LC-MS/MS. Peptide from the 30 and 60 hydrolysis was dominated by more hydrophobic peptide in 6-10 min retention time.

According to Zhao et al. (2013), hydrophobicity of the peptide is correlated to antimicrobial activity since peptide with higher hydrophobicity resulted higher activity. Peptide from goat milk protein hydrolyzed by protease *Bacillus* sp. E.13 for 30 and 60 minutes with high antimicrobial is dominated by hydrophobic peptide that is able to inhibit bacterial growth up to 5 log cycle. Althogh peptide with high hydrophobicity commonly showed high toxcicity, more over amino acid composition and distribution of the positive charge also

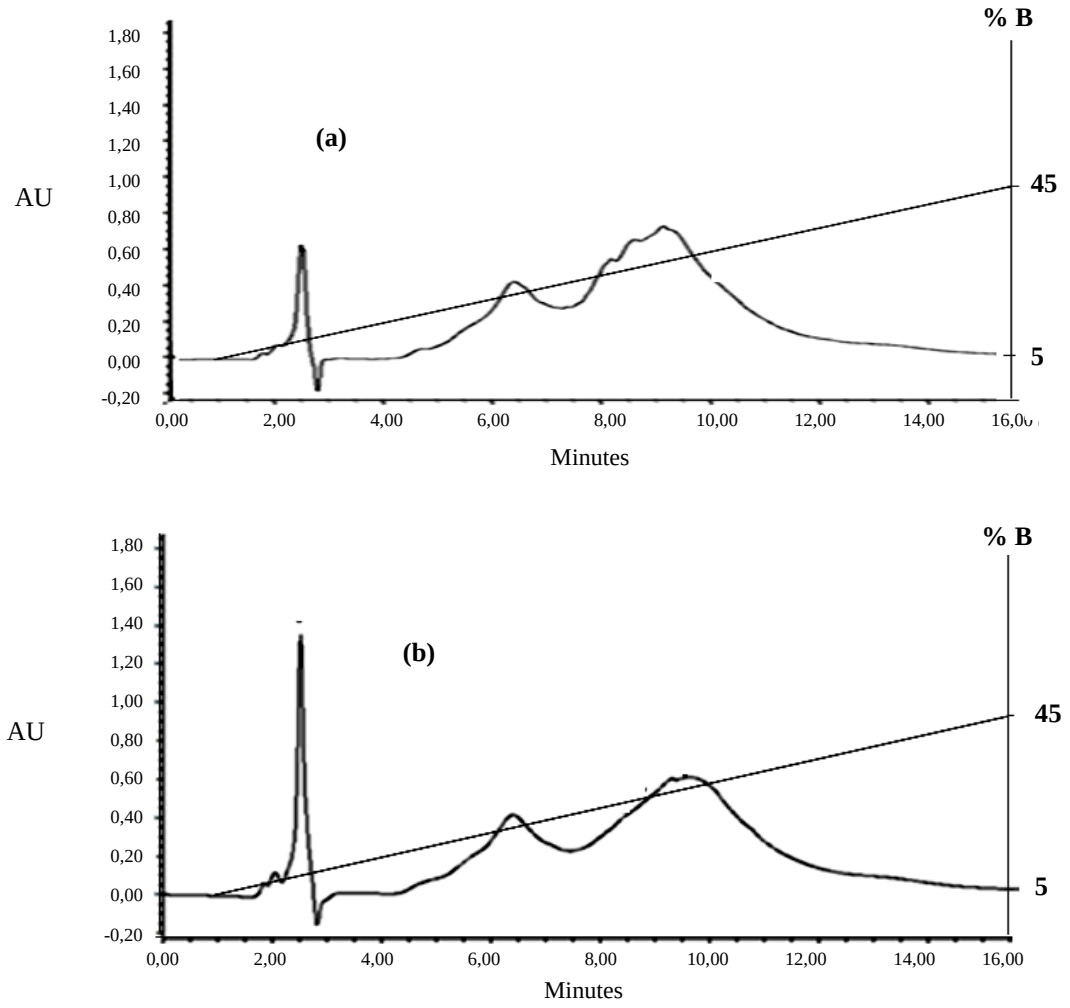

Figure 3. Profile of chromatogram of goat milk hydrolyzed by protease *Bacillus* sp at 55o C pH 11 for 30 minutes (a) and 60 minutes (b), separation was done using RP-HPCL with gradient 0,1% TFA in deionized water (A soluble) and 0,1% trifluoroacetic acid (TFA) in acetonitrile (ACN) (B soluble) with water flowrate by 1µl/minute at λ 215 nm

influence the toxicity determinant Yin et al (2012). For antioxidant activity, Ajibola et al. (2011) revealed that increasing hydrophobicity was related to antioxidant activity.

Peptides produced in this study may have multifunction as antimicrobial and antioxidant. Those peptides were highly potential to be applied to reduce the application of both antimicrobial drug and antioxidant suplement. The peptides were able to neutralize free radical excess due to interaction between peptides and bacteria cell. If the animal cell is infected by bacteria, interaction of animal and bacterial cells produces reactive oxygen species (ROS). High ROS concentration destroy host animal cells, even in excess condition cause the death (Sharma et al. 2012; Liu et al. 2013). Antimicrobial peptide such as diptericin had an important role in reducing ROS (Zhao et al. 2011). Antimicrobial peptide which is simultaneously has antioxidant activity is observed in the amphibian skin. That peptide killed microbes in a short time and neutralized free radical formed in seconds (Yang et al. 2009). That kinetic makes the amphibian refuge from infection and oxidative stress that may occur. The multifunction activities of our peptide has to be optimized to be applied for feed supplement. Purification of the peptide might increase the activity.

CONCLUSION

Peptide from goat milk protein hydrolyzed by *Bacillus sp*. E.13 protease showed antibacterial and antioxidant activities. The peptides were able to inhibit *L. monocytogenes*, *S. Typhimurium* and *E. coli* up to 5 log cycles and inhibit *S. aureus* up to 1 log cycle. The

ability of the peptide as antimicrobial and antioxidant very potent to be applied as feed supplement.

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Effect of Enzyme Supplementation on Nutritive Values of Fermented Palm Kernel Cake Used to Substitute Soybean Meal in Broiler Diet

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ABSTRAK

Sinurat AP, Purwadaria T, Purba M. 2015. Pengaruh penambahan enzim terhadap nilai gizi bungkil inti sawit terfermentasi untuk menggantikan bungkil kedelai dalam ransum ayam broiler. JITV 20(3): 184-192. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1185

Suatu penelitian dirancang untuk meningkatkan nilai gizi bungkil inti sawit (PKC) dengan proses fermentasi yang diikuti dengan penambahan enzim untuk menggantikan penggunaan bungkil kedelai dalam ransum ayam broiler. Percobaan pertama menggunakan rancangan faktorial 2 x 2 untuk mengetahui pengaruh proses fermentasi (PKC dan PKC terfermentasi atau FPKC) dan pengaruh penambahan enzim (tanpa enzim dan ditambah enzim BS4). Kecernaan bahan kering, energi metabolis (AME) dan kecernaan asam amino (IAAD) keempat bahan tersebut diukur dengan menggunakan ayam broiler jantan. Sebanyak 7 ulangan digunakan untuk mengukur kecernaan bahan kering dan AME, sedangkan untuk IAAD hanya 3 ulangan. Percobaan kedua dirancang untuk menggantikan bungkil kedelai dengan FPKC yang ditambah dengan enzim BS4 (EFPKC). Empat jenis ransum percobaan, yaitu kontrol (tanpa EFPKC) dan penggantian 10%, 20 dan 40% bungkil kedelai dengan EFPKC disusun dengan kandungan gizi yang sama dan memenuhi kebutuhan gizi ayam broiler. Ransum diberikan pada ayam broiler umur 1 hingga 35 hari, masing-masing dengan 6 ulangan. Bobot badan, konsumsi ransum, FCR dan mortalitas diamati selama percobaan. Pada akhir penelitian, diukur persentase karkas, lemak abdomen, bobot hati dan rempela. Hasil menunjukkan bahwa fermentasi PKC meningkatkan kecernaan bahan kering ($P<0.05$) dan AME meskipun tidak nyata ($P>0.05$). Penambahan enzim tidak nyata ($P>0.05$) mempengaruhi kecernaan bahan kering dan AME. Fermentasi nyata ($P<0.05$) menurunkan IAAD beberapa asam amino essensil. Namun, penambahan enzim tidak nyata ($P>0.05$) mempengaruhi IAAD asam amino essensil. Penggantian bungkil kedelai dengan EFPKC menyebabkan penurunan konsumsi pakan dan pertumbuhan pada ayam broiler.

Kata Kunci: Bungkil Inti Sawit, Fermentasi, Enzim, Broiler, Bungkil Kedelai

ABSTRACT

Sinurat AP, Purwadaria T, Purba M. 2015. Effect of enzyme supplementation on nutritive values of fermented palm kernel cake used to substitute soybean meal in broiler diet. JITV 20(3): 184-192. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1185

Two experiments were designed to improve nutritional values of palm kernel cake (PKC) by biofermentation process, followed by enzyme supplementation to substitute soybean meal (SBM) in broilers diet. A factorial of 2 x 2 design was applied in the first experiment, i.e. fermentation process (non fermented PKC and fermented PKC) and enzyme supplementation (no enzyme and +BS4 enzyme). Dry matter (DM) digestibility, AME and amino acids ileal digestibility (IAAD) of the treatment ingredients were measured in broiler chickens. Seven replicate were applied for the DM and AME assays and 3 replicate for IAAD assay. Second experiment was designed to study the effect of SBM substitution with enzyme supplemented FPKC (EFPKC). Four diets were formulated, i.e., control diet without EFPKC, 10%, 20 and 40% SBM substituted with EFPKC. All diets were formulated to meet the nutrient requirements of broilers. Each diet was fed to broilers from 1 to 35 d. Body weight, feed consumption, FCR and mortalities were measured. Carcass yield, abdominal fat and weight of liver and gizzard were measured at the end of experiment. Results showed that fermentation of PKC increased the DM digestibility, the AME was also increased but not significant. Enzyme supplementation did not affect the DM digestibility and AME of PKC. Fermentation process significantly ($P<0.05$) decreased IAAD of some indispensable amino acids. However, supplementation of enzyme did not affect the IAAD of indispensable amino acids. Substitution of soybean meal with EFPKC reduced the feed intake and growth rate of broilers.

Key Words: Palm Kernel Cake, Fermentation, Enzyme, Broilers, Soybean Meal

INTRODUCTION

Modern chickens (broilers and layers) require high quality feed to support their rapid growth, high productivity and high efficiency in feed utilization. Soy bean meal (SBM) is one of good quality feedstuff that commonly used as a protein and or amino acids source in chickens diet. Its inclusion in broiler's diet varies between 15 to 30%, depends on the presence of other protein sources included in the diet. Even in countries

with no SBM production such as Indonesia, SBM is commonly used in poultry diet. Increases of national broiler production causes increases in importation of SBM and price of feed. Indonesia imported 3.069 million ton SBM in 2010 and 4.250 million ton in 2014 (USDA 2015). In order to achieve self sufficiency in feed ingredients, it is important to seek alternatives to limit or replace SBM with local feed ingredients.

Some studies have been reported to reduce SBM in broiler's diet with the more cheaper ingredients such as rapeseed meal (Taraz et al. 2006; Riyazi et al. 2008; Ciurescu 2009), sunflower seed meal (Shi et al. 2012), fermented cotton seed meal (Azman & Yilmaz 2005) and peanut meal (Dhawale 2005).

As a world leading producer of palm oil, Indonesia produces palm kernel cake (PKC)- a by product of palm kernel oil production in a large quantity. The Indonesian PKC production in 2014 was estimated 4.55 million ton (USDA 2015). Its nutritive values including its digestible amino acids have been reported by some authors (Nwokolo et al. 1977; Onwudike 1986; Sue & Awaludin 2005; Sundu et al. 2006; Sinurat et al. 2014). The protein, amino acids and the digestible amino acids of the PKC are much lower than the SBM. Replacing the SBM with PKC as such for a protein source in poultry diet will deteriorate their performances. Sinurat et al. (2014) reported that the PKC could be fermented with *Aspergillus niger* to increase its crude protein from 14.76% to 20.04%. Some amino acids (except threonine and arginine) content increased varies between 5.2-117.2% and also its protein and ileal- amino acids digestibility.

Although, the protein and amino acids contents and the ileal amino acids digestibility of the fermented PKC were higher than the PKC, it is still not comparable with those of the SBM. The feeding trial on laying hens showed that only 25-50% of the SBM could be substituted with enzyme supplemented fermented PKC without significantly affecting egg production and feed efficiency (Sinurat et al. 2014). In order to increase protein and amino acids content of PKC, Sinurat et al. (2015) have modified the fermentation method by adding cassava leaf meal as protein sources prior to PKC fermentation. This new method was able to increase protein content of fermented PKC from 21.91 to 28.97% as compared to the previous method (Sinurat et al. 2014). The methionine, lysine, tryptophan and threonine levels were also increased from 0.290 to 0.317%, 0.643 to 0.740, 0.150 to 0.273 and 0.31 to 0.273%, respectively.

Enzymes have been widely used to increase nutrients availability of poultry feedstuff or feed. Enzyme complex (consist of xylanase, beta-glucanase and cellulose) have been reported to increase the crude protein digestibility and the AME of feed containing paddy and rice bran (Kang et al. 2013). Khan et al. (2006) also reported an increase on the dry matter-, organic matter-, crude protein and energy-digestibilities of feed containing sunflower meal caused by supplementation of commercial enzymes. A multi enzyme produced by *Eupenicillium javanicum* has been developed in our laboratory (Purwadaria et al. 2003; Pasaribu et al. 2009). The enzyme, called BS4 has been reported effectively to improve nutrient digestibility of palm oil by products such as palm oil sludge (Pasaribu et al. 2009) and palm kernel cake (Sinurat et al. 2013). Sinurat et al. (2014) also showed that the enzyme improved the protein- and the amino acids-digestibilities of fermented PKC.

Therefore, an experiment was designed to test the biological values of the fermented PKC (produced with the new method) supplemented with the BS4 enzyme and the possibility of using the product to substitute SBM in broiler's diet as reported in this paper.

MATERIALS AND METHODS

Effect of enzyme supplementation on dry matter digestibility, AME and IAAD of PKC and fermented PKC (FPKC)

Two steps of experiments were carried out in order to test biological values of fermented PKC. The first experiment was to determine AME and ileal amino acids digestibility of the FPKC produced with new fermentation method as described by Sinurat et al. (2015). The PKC was pretreated by autoclaving and supplemented with 10 % cassava leaf meal (9:1) before fermented with *A. niger*.

For the digestibility (dry matter, protein, ileal amino acids digestibility and AME) study, a commercial broiler feed was used as basal diet (B). Basal diet was mixed with ingredients tested 50:50 and added 2% acid insoluble ash (celite) as an indicator as described in Ravindran et al. (2005) and Sinurat et al. (2014). The experiment was arranged in a 2 X 2 factorial design. The first factor was fermentation process, i.e., non fermented PKC and fermented PKC (FPKC) and the second factor was enzyme supplementation (no enzyme and with enzyme). The enzyme supplemented was BS4 enzyme (150 U/kg) as reported by Sinurat et al. (2014). Parameters measured were dry matter digestibility, apparent metabolisable energy (AME) and ileal amino acids digestibility (IAAD).

One hundred (100) broiler chicks were reared on litter pens with standard rearing management from one day to 28 days old. At 28 d, 35 male chicks were selected and placed in individual wire cages. Each treatment diet was fed ad libitum to 7 (seven) chicks, and considered as replications. Treatment diets were fed for 5 days and samples of excreta was collected at the last 3 days for AME determination. After 5 days

feeding the tested diet, 35 birds were sacrificed by CO_2 asphyxiation and the digesta in the ileal was collected into plastic containers. Samples from 2 or 3 birds fed with the same diet were pooled to make 3 sample replications for each treatment. The digesta were immediately kept in the freezer for further chemical analyses.

The feed and the tested ingredients were analysed for dry matter, nitrogen (protein), amino acids and acid insoluble ash (AIA) contents. The excreta collected was analysed for the dry matter, nitrogen (protein), gross energy and AIA contents while the ileal digesta were analysed for dry matter, amino acids and AIA contents. The dry matter and the nitrogen were analysed following procedures described by AOAC (2005), while the amino acids were analysed by HPLC method at Bogor Agricultural University laboratory. All data were subject to analyses of variance, followed by least significance difference when the ANOVA was significant ($P<0.05$) according to procedure described by Steel & Torrie (1997).

Table 1. Composition of experimental diets in the feeding trial

	Control	SBM-10	SBM-20	SBM-40
Ingredients (%)				
Maize	62.18	58.78	57.58	57.54
Soy bean meal	26.85	24.17	21.2	14.27
Meat and bone meal	3.93	3.3	3.83	7.0
Corn gluten meal	3.0	3.0	3.0	3.0
Limestone	0.47	0.54	0.44	0.31
Crude palm oil	1.5	2	2	1.5
Fermented PKC + Enzyme	0	5.9	9.67	14.5
DL Methionine	0.35	0.37	0.3	0.43
L-lysine	0.35	0.41	0.46	0.55
Threonine	0.07	0.1	0.13	0.18
Tryptophan	0	0	0.01	0.05
Mono calcium phosphate	0.76	0.9	0.76	0.1
Sodium bicarbonat	0.1	0.1	0.1	0.1
Vitamin mix	0.025	0.025	0.025	0.025
Mineral mix	0.05	0.05	0.05	0.05
Salt	0.2	0.2	0.2	0.2
Choline Chloride	0.1	0.1	0.1	0.1
Coccidiostat	0.05	0.05	0.05	0.05
Fungistat	0.05	0.05	0.05	0.05
Total	100.0	100.0	100.0	100.0
Nutrient composition				
Crude protein, %	21.8	21.8	21.8	21.8
Metabolisable energy, kcal/kg	3000	3000	3000	3000
Digestible lysine, %	1.185	1.185	1.185	1.185
Digestible methionine, %	0.633	0.665	0.679	0.697
Digestible meth. + cys, %	0.908	0.905	0.905	0.905
Digestible tryptophan, %	0.208	0.202	0.202	0.202
Digestible threonine, %	0.758	0.758	0.758	0.758
Calcium, %	0.90	0.90	0.90	1.05
Available P, %	0.46	0.46	0.46	0.46

Substitution of soybean meal with enzyme supplemented fermented palm kernel cake (EFPKC) in broilers diet

The second experiment was designed to study the effect of substitution of SBM with the EFPKC on the performance of broilers. Four dietary treatments with graded levels of SBM substitution with EFPKC were formulated. All diets were formulated to meet the nutrient requirement of broilers according to Cobb (2012), i.e., crude protein 21.8%, metabolizable energy 3000 kcal/kg, digestible amino acids (lysine 1.185%, methionine + cystine 0.905%, tryptophan 0.202%, threonine 0.758), calcium 0.90% and available phosphorus 0.46%. The ingredients and nutrient composition of the experimental diets are shown in Table 1.

The nutrient values of the EFPKC (ME and digestible amino acids) obtained from experiment 1, was used for the formulation of the treatment diets. The treatments consist of:

1. Standard diet (Control = C) with SBM level as in normal broiler diet
2. Diet with 10% of the SBM substituted with EFPKC
3. Diet with 20% of the SBM substituted with EFPKC
4. Diet with 40% of the SBM substituted with EFPKC

Each diet was fed to 60 broilers (6 replicates and 10 birds per replicate) from 1 to 35 days old. The birds were reared in pens with rice hulls as litter. Feed and water were given ad libitum. Parameters observed were feed intake, body weight, mortalities, and feed convertion ratio. At the end of the feeding trial, 2 birds (1 male and 1 female) from each pen were slaughtered to determine carcass and abdominal fat. Data were analysed with analyses of variance (ANOVA) in a completely randomized design (4 treatments X 6 replicates). Duncan's multiple range test were applied to show difference between treatment means when the ANOVA was significant at $P<0.05$ (Steel & Torrie 1997).

RESULTS AND DISCUSSION

Nutrients digestibility of PKC and FPKC as affected by enzyme supplementation

Effect of fermentation process and BS4- enzyme supplementation on dry matter digestibility and metabolisable energy (AME) of PKC and FPKC are presented in Table 2. The dry matter (DM) digestibility of PKC was only significantly ($P<0.05$) affected by fermentation (F) process, but not by enzyme supplementation (E) nor by the interaction of F x E. The DM digestibility of PKC in this study was 37.3% while previous study (Sinurat et al. 2013) showed a higher DM digestibility, i.e. 56.8%.

The AME of the PKC was not significantly ($P>0.05$) affected by fermentation process (F), enzyme supplementation (E) nor by the interaction of F x E. The AME of the PKC obtained in this experiment was 2079 kcal/kg, almost similar to the previous study i.e., ME 2074 Kcal/kg (Sinurat et al. 2014) and 2091 kcal/kg (Sinurat et al. 2013). However, Saenphoom et al. (2013) reported a lower TME of PKC i.e., 4.71 MJ/kg (or 1126 kcal/kg), which may be due to different process applied in the production of the PKC.

Some reports have shown different results on the effect of fermentation process on metabolisable energy of PKC. Sinurat et al. (2014) showed that fermentation of PKC with *A. niger* significantly decreased the AME (from 2074 to 1788 kcal/kg). Similar patterns were also reported by Muangkeow & Chinajariyawong (2009) after fermented with *A. wentii.* Dairo & Fasuyi (2008) also reported a decrease in the ME of PKC after fermentation without addition of a fungus as inoculum. In contrast, Bintang et al. (1999) and Iyayi & Aderolu (2004) showed an increase in the ME of PKC after fermented with *A. niger* and *Trichoderma viride*, respectively.

In this study, PKC was mixed with 10% cassava leaf meal (CLM) prior to fermentation as described by Sinurat et al. (2015). Despite the ME of CLM, (i.e. 1160 kcal/kg according to Darma et al. 1989), was much lower than the ME of PKC, the fermentation process was still able to increase the ME of the PKC, although the differences were not significant ($P>0.05$).

Enzyme supplementation did not significantly ($P>0.05$) affect the DM digestibility nor the AME of the PKC nor the FPKC, although it increased from 37.3% to 46.8% or 25.5% improvement. Previous study showed a similar but significant improvement (26.2%) in the DM digestibility of the PKC due to enzyme supplementation (Sinurat et al. 2014). The DM digestibility of the PKC was increased from 56.8% to 71.7% as affected by the enzyme supplementation. Although statistically not significant, the AME of the PKC was increased from 2079 to 2385 kcal/kg or 15% improvement, while previous study showed an increase from 2091 to 2317 kcal/kg or 11% improvement. The AME of the FPKC was only slightly increased as the effect of enzyme supplementation, i.e., from 2496 to 2554 kcal/kg or 2.3% improvement. Previous study (Sinurat et al. 2014) showed a similar trend, i.e., 4.4% improvement due to addition of similar enzymes. Saenphoom et al. (2013) showed a high (60.3%) improvement on the TME of PKC as effect of enzymes (cellulase and mannanase) supplementation. In their study, the PKC was soaked in water, added with the enzyme, and incubated for 18 hours before the digestibility study. Present results showed that the enzyme improved DM digestibility and AME less on fermented PKC as compared to the effect on non-

Table 2. Dry matter digestibility and metabolisable energy of PKC and FPKC fermented PKC with enzyme supplementation

Ingredients	Dry matter (DM) digestibility, %	Metabolisable energy (AME), kcal/kg
Palm kernel cake (PKC)	37.3[b*]±12.5**	2079±389[a]
PKC + BS4 enzyme	46.8±4.35[ab]	2385±152[a]
Fermented PKC (FPKC)	50.64±9.1[a]	2496±371[a]
FPKC + BS4 enzyme	51.4±13.6[a]	2554±511[a]
Significance (P)		
Fermentation (F)	0.045	0.12
Enzyme (E)	0.251	0.39
F x E Interaction	0.255	0.63

* Values in the same column with different superscript significantly different (P<0.05)
** Standard deviation. Coeficient variation (CV) of the DM digestibility: 22.14%; AME: 14.8%

fermented PKC. Less increase of AME due to the enzyme supplementation to FPKC compared to PKC might occur due to the saccharification activity of fibernolytic enzymes produced during fermentation. The FPKC may contain shorter fiber molecules than that of PKC, therefore they were less digested by the enzyme addition. Purwadaria et al. (1998) reported that fermented palm oil sludge with *A. Niger* produced mannanase and cellulase in the course of fermentation. Those enzymes might actively digest the fiber.

Although the DM digestibility and the AME were increased due to fermentation process and enzyme supplementation, the effects were not significant (P>0.05) statistically. Perhaps, this is due to the high variability on the parameter observed in this study as shown by the coefficient of variation (CV), i.e., 22.14% and 14.8% for the DM digestibility and AME, respectively (Table 2).

The level of digestible amino acids in a feedstuff is calculated by multiplying the amino acids concentration in the feedstuff with the digestibility coefficient (percentage) of the amino acids. These values are commonly used for poultry ration formulation. The digestible amino acids of the PKC and FPKC as affected by enzyme supplementation are presented in Table 4.

Effect of enzyme supplementation on ileal amino acids digestibility (IAAD) of PKC and FPKC is presented in Table 3. The IAAD of essential (or indispensable) amino acids were not significantly (P>0.05) affected by enzyme supplementation nor by interactions between fermentation process (F) and enzyme supplementation (E). Some indispensable amino acids, i.e., arginine, histidine, isoleucine, leucine, phenylalanine and valine were significantly (P<0.05) decreased by fermentation process. These results do not agree with the results of Muangkeow & Chinajariyawong (2009) which showed that true amino acids (except for arginine) digestibility of the PKC were increased after fermented with *A. wentii.* Sinurat et al. (2014) also reported an increase in IAAD of indispensable amino acids except the arginine, threonine, tryptophan and valine. Supplementation of cassava leaf meal in PKC fermentation process may have changed IAAD profiles of FPKC. The IAAD values of PKC obtained in this study were much higher than IAAD of the PKC used in the previous experiment (Sinurat et al. 2014) which indicate the difference in quality of raw material (PKC) used in fermentation.

Among the indispensable amino acids, only digestible arginine, lysine and threonine were affected by treatments significantly (P<0.05). The digestible arginine level was significantly (P<0.05) reduced by the fermentation (F), but not significantly (P>0.05) affected by the enzyme (E) supplementation nor by their interaction (F x E). The digestible lysine was significantly (P<0.05) affected by the interaction (FxE), in which the enzyme supplementation increased the digestible lysine in PKC but not significantly (P>0.05) affected when applied in the FPKC. The digestible threonine was significantly (P<0.01) higher in the FPKC as compared to the PKC. In general, data on Table 4 showed that supplementation of enzyme did not increase the digestible amino acids in the FPKC. Solid substrate fermentation of palm kernel cake using *A. niger* USM F4 produced mannanase, a hemicellulytic enzyme for digesting NDF in PKM (Syarifah et al. 2012). Since the fermentation process of PKM in this experiment also produced enzymes, therefore supplementation of exogenous enzyme may have been in excess. As shown by Pourreza et al. (2007) supplementation of enzymes to a certain levels or activities improves the nutrients (dry matter, energy and protein) digestibility of feed, but supplementation of enzyme in a higher levels did not. Their data also showed that excess exogenous enzymes supplementation reduced nutrients digestibility of the feed.

Table 3. Ileal amino acids digestibility of PKC and fermented-PKC with enzyme supplementation (%)

Amino acids	Basal*	PKC	PKC+ Enzyme	FPKC	FPKC+ Enzyme	Significance (P)		
						Fermen-tation(F)	Enzyme (E)	F x E
Indispensable amino acids								
Arginine	93.50	92.86	87.44	76.68	77.70	0.005	0.538	0.374
Histidine	89.28	87.11	70.35	56.55	65.86	0.016	0.537	0.054
Isoleucine	88.97	91.03	84.02	75.56	74.37	0.013	0.327	0.480
Leucine	90.67	91.77	84.08	77.31	76.89	0.023	0.325	0.375
Lysine	91.79	67.34	78.09	72.21	72.41	0.921	0.204	0.219
Methionine	93.05	87.18	75.24	81.05	83.69	0.893	0.594	0.410
Phenylalanine	90.15	95.13	84.43	73.52	74.78	0.005	0.285	0.184
Threonine	86.41	82.45	72.51	68.58	71.61	0.109	0.424	0.153
Valine	89.22	86.91	80.48	72.06	72.16	0.024	0.470	0.456
Dispensable amino acids								
Aspartic acid	85.15	58.15	67.80	67.05	75.61	0.108	0.085	0.908
Glutamic acid	88.94	78.49	81.41	74.79	75.93	0.258	0.604	0.820
Glycine	88.18	83.65	71.63	75.50	66.72	0.132	0.028	0.689
Proline	93.92	95.48	89.95	83.82	77.85	0.003	0.080	0.941
Serine	88.03	83.91	74.09	68.46	74.68	0.067	0.621	0.052
Tyrosine	88.38	90.76	70.05	58.49	60.12	0.032	0.278	0.208

*Basal diet is a commercial broiler starter diet. The data were used for calculation of the IAAD

Table 4. Digestible amino acids of PKC and fermented- PKC with enzyme supplementation (%)

Amino acids	PKC	PKC+ Enzyme	FPKC	FPKC+ Enzyme	Significance (P)		
					Fermen-tation(F)	Enzyme (E)	F x E
Dispensable amino acids							
Arginine	1.494	1.443	1.337	1.293	0.009	0.316	0.936
Histidine	0.334	0.288	0.302	0.308	0.764	0.332	0.216
Isoleucine	0.732	0.702	0.755	0.708	0.630	0.210	0.780
Leucine	1.139	1.081	1.190	1.131	0.285	0.218	0.991
Lysine	0.436	0.532	0.614	0.582	0.002	0.234	0.033
Methionine	0.255	0.231	0.282	0.279	0.125	0.551	0.631
Phenylalanine	0.809	0.743	0.821	0.789	0.400	0.165	0.626
Threonine	0.261	0.246	0.537	0.518	<0.001	0.413	0.943
Valine	0.925	0.896	0.958	0.904	0.613	0.320	0.762
Dispensable amino acids							
Aspartic acid	0.982	1.243	1.426	1.453	0.002	0.073	0.132
Glutamic acid	2.415	2.618	2.562	2.459	0.967	0.625	0.156
Glycine	1.508	1.378	0.691	0.594	<0.001	0.010	0.636
Proline	1.475	1.419	0.426	0.389	<0.001	0.014	0.552
Serine	0.613	0.574	0.551	0.551	0.056	0.344	0.336
Tyrosine	0.404	0.334	0.485	0.452	0.044	0.250	0.675

Table 5. Performances of broilers as affected by substitution of soybean meal with fermented palm kernel cake supplemented with enzyme

Parameters	Control	10% SBM Substituted	20% SBM Substituted	40% SBM Substituted	Significance (P)
DOC body weight, g	42.4	43	42.8	43.9	0.857
Bodyweight 21 d, g	859.0	815.5	842.5	821.9	0.850
Bodyweight 35 d, g	1929.6[a]	1616.9[b]	1753.2[b]	1699.8[b]	0.008
Feed intake 1 – 21 d, g	1331.7[a]	1149.6[b]	1250.6[a]	1143.0[b]	<0.001
Feed intake 1 – 35 d, g	3116[a]	2831.2[b]	3026.5[ab]	2825.5[b]	0.043
FCR 1 – 21 d	1.401	1.488	1.487	1.504	0.158
FCR 1 – 35 d	1.657[b]	1.804[a]	1.743[ab]	1.706[b]	0.017
Mortalities, %	3.33	6.67	10.0	16.7	0.059

*Different superscript in the same row showed significant difference (P<0.05)

Tabel 6. The effect of substitution of soybean meal with enzyme fermented palm kernel cake on the carcass percentage and some organs weight

Parameters	Control	10% SBM Substituted	20% SBM Substituted	40% SBM Substituted	Significance (P)
Dressed carcass, % BW	77.20	76.00	76.50	77.70	0.413
Abdominal fat, g/kg BW	19.86	24.09	23.65	22.19	0.139
Liver weight, g/kg BW	14.18	12.24	15.95	15.23	0.081
Gizard weight, g/kg BW	19.23	22.25	19.84	19.97	0.262

Effect of substitution of soybean meal (SBM) with the enzyme supplemented FPKC (EFPKC) on performance of broilers from day old to 35 days old is presented in Table 5. The feed intake of broilers at 1-21 d old was significantly (P<0.01) affected by treatments. The lowest feed intake was found when the SBM was substituted 10%, followed by 40% substitution. The feed intake was different significantly (P<0.05) between control (not substituted) with 10% and 40% substitution of SBM with EFPKC, however, substitution of 20% SBM did not show significant (P>0.050) difference with the control. Similar trend and significant (P<0.05) effect was also found on the feed intake during 1-35 d period. Since all dietary treatments were formulated with similar nutrient values, include the energy (ME) and the digestible amino acids, the difference on feed intake could not be due to the nutrient factors but may be there are some anti-nutrient substances in the EFPKC. If this assumption is true, then the more the SBM substituted, the more the EFPKC included in the diet. If the EFPKC contains some anti-nutrient substances, the lowest feed intake was expected to occur when 40% SBM was substituted with the EFPKC. This phenomenon has been reported by Sinurat et al. (2014) when the SBM was substituted with the fermented PKC (without enzyme supplementation) in laying hens diet. It could not be explained at this stage why this is not the case in this experiment.

Body weight of broilers at 21 d was not significantly (P>0.05) affected by the substitution of SBM with EFPKC. However, the body weight at 35 d was significantly (P<0.05) affected by the treatments. Substitution of SBM with EFPKC produced lighter birds at 35 d, as compared with the control. The body weight is a reflection of feed or nutrients intake. The heavier birds were found on control diet (859.0 g at 21 d and 1929.6 g at 35 d) which consumed the highest feed and the body weight was lighter as the feed intake reduced.

The feed convertion ratio (FCR) during 1 to 21 d was not significantly (P>0.05) affected by treatments, however the FCR during 1-35 d was significantly (P<0.05) affected. The FCR data showed that birds fed with the control diet were the most efficient in feed utilization. However, statistical analyses showed the FCR was only significantly different (P<0.05) between birds fed the control diet and those fed with 10% SBM substitution.

The mortalities of birds during the experiment was not significantly (P>0.05) affected by treatments. The data, however, showed that the mortalities increased as the level of SBM substitution with EFPKC was increased.

The effect of substitution of SBM with EFPKC on carcass percentage, weight abdominal fat, liver and gizzard of broilers as affected by treatments is presented in Table 6. Statistical analyses showed that there was no significant (P>0.05) effect of treatments on the dressed carcass percentage, abdominal fat-, liver- and gizzard-weight of broilers. These results indicated that replacing the SBM with EFPKC did not change the metabolism of the birds.

CONCLUSION

This study concludes that fermentation process improved the nutrient values of the palm kernel cake. Supplementation of enzyme was not effective to improve AME and amino acids digestibility of fermented palm kernel cake. Supplementation of enzymes to fermented palm kernel cake was not recommended to substitute soybean meal in broiler diets, since it reduced feed intake and growth rate of broilers.

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Administration of Extract *Salix tetrasperma* Combined with Extract of Turmeric and Neem to Improve Eggs Quality of Chicken Reared under Heat Stress

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ABSTRAK

Sugito, Hambal M, Isa M, Nurliana, Delima M. 2015. Pemberian ekstrak *Salix tertrasperma* kombinasi dengan ekstrak kunyit dan mimba untuk peningkatan kualitas telur ayam yang dipelihara pada suasana stres panas. JITV 20(3): 193-199. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1186

Kualitas telur akan menurun apabila ayam mengalami stres panas. Pemberian beberapa jenis ekstrak tanaman dilaporkan dapat mengurangi dampak tersebut. Tujuan penelitian ini adalah untuk mengetahui efek pemberian ekstrak *Salix tetrasperma* dikombinasikan dengan ekstrak kunyit dan mimba untuk meningkatkan kualitas dan produktivitas ayam petelur dalam kondisi stres panas. Penelitian ini menggunakan 60 ekor ayam petelur strain Isa Brown periode layer berumur 6 bulan. Pemeliharaan dilakukan pada kandang kawat individu. Pemberian pakan dan minum secara ad libitum. Pelaksanaan penelitian menggunakan metode rancangan acak lengkap dengan 5 perlakuan dan masing-masing perlakuan terdiri atas 12 ulangan. Sebagai kontrol negatif tanpa diberi suplemen antistres (KN) dan kontrol positif diberi suplemen antistres komersil (KP). Formulasi dosis ekstrak yang diberikan adalah *S. tetrasperma* 1.000 mg/l air minum (EJ), *S. tetrasperma* 1.000 mg/l + kunyit 250 mg/l + mimba 250 mg/l (EJ+K1), dan *S. tetrasperma* 1.000 mg/l + kunyit 500 mg/l + mimba 500 mg/l air minum (EJ+K2). Ayam dipelihara dalam suasana stres panas selama 5 jam perhari pada kisaran suhu 34,0 ± 1,5°C. Pemberian suplemen dilakukan dengan cara dilarutkan dalam air minum dan diberikan selama 30 hari pada pagi sampai siang hari. Hasil penelitian menunjukkan bahwa pemberian ekstrak *S. tetrasperma* secara tunggal ataupun dikombinasi dengan ekstrak kunyit dan mimba mempengaruhi (P<0,05) tebal kerabang telur, tetapi tidak mempengaruhi warna kuning telur, tinggi albumin, berat telur, dan nilai Haugh Unit (HU). Pemberian ekstrak *S. tetrasperma* 1.000 mg/l air minum dapat digunakan untuk meningkatkan kualitas telur pada ayam yang menderita stres panas.

Kata Kunci: Kualitas Telur, *Salix tetrasperma*, Kunyit, Mimba, Stres Panas

ABSTRACT

Sugito, Hambal M, Isa M, Nurliana, Delima D. 2015. Administration of extract *Salix tetrasperma* combined with extract of turmeric and neem to improve eggs quality of chicken reared under heat stress. JITV 20(3): 193-199. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1186

Quality of eggs might decrease when hens under heat stress. A further study found that a specific plant extracts could reduce the impacts of heat stress. The aim of this study was to determine effects of *Salix tetrasperma* plant extract in combination with extract of turmeric and neem to improve egg quality and productivity of laying hens under heat stress. Sixty laying hens strain Isa Brown of 6 months old were used and reared in individual cages. The feed and drinking water were supplied ad libitum. This study was conducted in a completely randomized design with five treatments (two controls and three treatments) and each treatment consisted of 12 replication. Treatment consisted of with (KP) and without (KP) commercial anti-stress supplement. Formulations of extract were *S. tetrasperma* 1.000 mg / l water (EJ), *S. tetrasperma* 1.000 mg / l + Turmeric 250 mg / l + neem 250 mg / l (EJ+K1), and *S. tetrasperma* 1.000 mg / l + Turmeric 500 mg / l + neem 500 mg / l drinking water (EJ+K2). The hens were exposed to heat stress for 5 hours per day at a temperature range of 34.0±1.0°C. Supplements were dissolved in drinking water and were given for 30 days in the morning and noon. Results showed that a single extract of *S. tetrasperma* or the combination of an extract of turmeric and neem were significantly increased thickness of eggshell (P<0.05), but did not affect color of egg yolk, height albumin, egg weight, and HU value. Extract of *S. tetrasperma* combined with turmeric and neem extract dissolved in drinking water for 30 days in laying hens reared under heat stress could not improved quality of the eggs, but may increase thickness of the egg shell and cause decreased water consumption.

Key Words: Egg Quality, *Salix tetrasperma*, Turmeric, Neem, Heat Stress

INTRODUCTION

Daily environment temperature in several areas in Aceh Province is relatively high. During the day in the dry season, temperature is around 31-35ºC. Ahmadi & Rahimi (2011) said that temperature and humidity in an area became a critical factor affecting laying hen production. Laying hens kept out of their thermos-neutral condition experience physiology change and decreasing their egg production and quality. According to Talukder et al. (2010) and Feizi et al. (2012), optimum environment temperature for laying hen productivity was around 15-27ºC with relative humudity around 60-70%. Higher temperature and humidity than 27ºC and 70% respectively causes heat stress. Heat stress decreases body weight, egg production, consumption, egg weight, and egg tickness of laying hen.

Herbs used may decrease heat stress impact in laying hen. Previous research showed that administration of *Salix tetrasperma* Roxb plant extract in broiler chicken may decrease heat stress impact (Sugito et al. 2006). The last research result showed than administration of *Salix tetrasperma*, both as a single or combined with extract of tumeric and neem may be used to decrease heat stress impact and did not affect health status of laying hen (Sugito et al. 2014).

Attempt to improve potential of *S. tetrasperma* extract by formulating it with other plant may decrease heat stress impact. Tumeric and neem were chosen supported by fact that administration of turmeric or neem may decrease negative impact of heat stress in chicken, as reported by Riasi et al. (2012) that supplementation with turmeric meal by 0.5-2 g/kg diets may improve egg quality and productivity. Nadia et al. (2008) also reported that administration of turmeric meal of 0.5% of diet may be used as antioxidant in laying hen. Moreover, Esonu et al. (2007) reported that administration of neem up to 15% may increase egg production and yolk color.

Dey et al. (2011) said that administration of diet contained neem meal by 10-15g/kg in laying hen may increase albumen index and had positive quadratic impact in Haugh Unit (HU). Decrease of heat stress impact is related to bioactive compound in plants. The main bioactive compound of the *S. tetrasperma* is salicylic acid, curcumin in the turmeric and nimbidin in the neem plant and those derivatives. By formulating those 3 plants, it is be expected that afication of the bioactive compound will has better synergy impact to decrease heat stress impact in laying hen.

This study was aimed to determine effect of administration of *S. tetrasperma* extract single or combined with extract of turmeric and neem to improve quality and egg productivity of laying hen experienced heat stress.

MATERIALS AND METHODS

Laying hens

Sixty 6 months old laying hens strain Isa Brown were used in this study. Those hens were kept in individual wire cage. Diet and drinking water were given ad libitum. Before treated, the hens were adapted for 1 week. This study was done based on completely randomized design with 5 treatments and 12 replications for each treatment.

Treatments

Treatments given was showed in Table 1. Anti-stress supplement was dissolved in the drinking water and given for 30 days in the morning and noon. Administration and replacement of diets was done in the noon. Diet fed was commercial feed code 234-1.

Hens were kept in cage temperature was increased up to 32-34ºC for 5 hours/day for 30 days to inflict heat stress condition. Temperature and humidity were controlled using digital thermometer. To get the temperature range, eight 100 watt bulbs were lighted. Fan was used to remove heat. Lighting used 20 watt neon lamps lighted for 10 hours/day.

Table 1. Treatment of supplement administered to laying hens with extract of *S. tetrasperma* leaf, turmeric rizhome, and neem leaf

Extract Material	Control (-) (KN)	Control (+) (KP)	Extract of *S. tetrasperma* (EJ)	Extract of *S. tetrasperma* + Combination 1 (EJ+K1)	Extraxt of *S. tetrasperma* + Combination 2 (EJ+K2)
Commercial anti-stress	-	5 mg/lt	-	-	-
S. tetrasperma	-	-	1000	1000	1000
Turmeric	-	-	-	250	500
Neem	-	-	-	250	500

Extraction of *S. tetrasperma* leaf, turmeric rhizome, and neem leaf

Extraction of *S. tetrasperma* leaf, turmeric rhizome, and neem leaf were conducted by maceration method. *S. tetrasperma* leaf, turmeric rhizome, and neem leaf were cleaned, dried, and powdered before it was macerated. Each simplesia material was macerated 2 times by ethanol 70%. Filtrate obtained was concentrated using rotary evaporator to be condensed extract. The condensed extracts were made with each concentration as in Table 1 processed to be effervescent blend with material as follow: citric acid, tartaric acid, sodium bicarbonate, binder, and emulsifier of carbon-methyl-cellulose (CMC). Its administration was dissolved in the drinking water.

Parameters

Variables measured were egg production for 30 days, diet consumption, consumption of water added by supplement, yolk color, albumen height, egg weight, Haugh Unit (HU) value, and eggshell thickness. Assessment of yolk color, albumen height, egg weight, and HU value were done using Egg Analyzer (Made in Japan). Egg Shell Thickness Gauge (Orka Technology Ltd) was used to measure eggshell thickness.

Statistical analysis

Variables observed was analyzed by ANOVA with significant level by 5%. Significant effect from the treatment was further tested by Duncan multiple range test.

RESULTS AND DISCUSSION

Environment condition while research

During this study, temperature outside and inside of cage and daily humidity was presented in Figure 1. The highest temperature outside of cage at around 12.00-14.00 was around 35.6-36.0°C. Average temperature and humidity inside the cage were around 33.7-34.1°C and 58.4-50.1% respectively. Those temperature and humidity were in danger category (THI range= 83-86) shown by behavior change of whole hens such as panting. According to Muchacka et al. (2012), in chicken experiencing heat stress, behavior changes will be seen. Those changes are effort to eliminate or decrease heat stress in their body. Clinically, in chicken experienced heat stress experience increase in respiratory (panting). Panting indicated that chicken used in this study suffered heat stress.

Hens performance and egg quality

Average egg production, diet and water consumption, and ratio of feed conversion of laying hens during this study were presented in Table 2. Administration of *S. tetrasperma* and its combination was not significantly ($P>0.05$) affect egg production. In the EJ+K1 was a treatment with highest egg production (reaching 99.4%) with the lowest RKP value compared to the other treatments. Addition of *S. tetrasperma* extract combined with extract of turmeric and neem by each became 500 mg/l of drink water in fact may not affect egg production, diet consumption, and ratio of feed conversion, but significantly ($p<0.01$) affecting water consumption. Result of comparative test showed that administration of *S. tetrasperma* extract combined with extract of turmeric and neem may decrease ($P<0.05$) water consumption. The most water consumption in hens treated by EJ and the lowest one was in EJ+K2 treatment (Table 2).

Impact of heat stress to egg production, yolk color value, albumen height, HU value, and RKP value in hens has not been seen, both of Negative Control (Table 1 and 2) or addition of *S. tetrasperma* extract and its combination. This is in contrast to Mashaly et al. (2004) and Kilic & Simsek (2013) reported that heat stress in laying hens lead to decrease of production and quality of egg and diet consumption. Allahverdi et al. (2013) said that heat stress in chicken caused increase of free radical compound, disturbance of acid-base balance, and calcium metabolic disturbance. This physiology change affected production and quality of egg.

Administration of *S. tetrasperma* extract as single (EJ treatment) did not cause decrease of water consumption, instead the number of water consumed was more than those of EJ+K1 and AJ+K2. This was suspected that administration of *S. tetrasperma* did not affect taste of the drinking water. In contrast to that, administration of *S. tetrasperma* combined with extraxt of turmeric and neem caused decrease of water consumption. That decrease in those EJ+K1 and EJ+K2 treatments was suspected due to the water was relatively bitter than those KN, KP, and EJ treatments. Kudo et al. (2010) said that laying hen was a sensitive bird to bitter taste. Bitter taste in the diet and drinking water caused decrease in consumption.

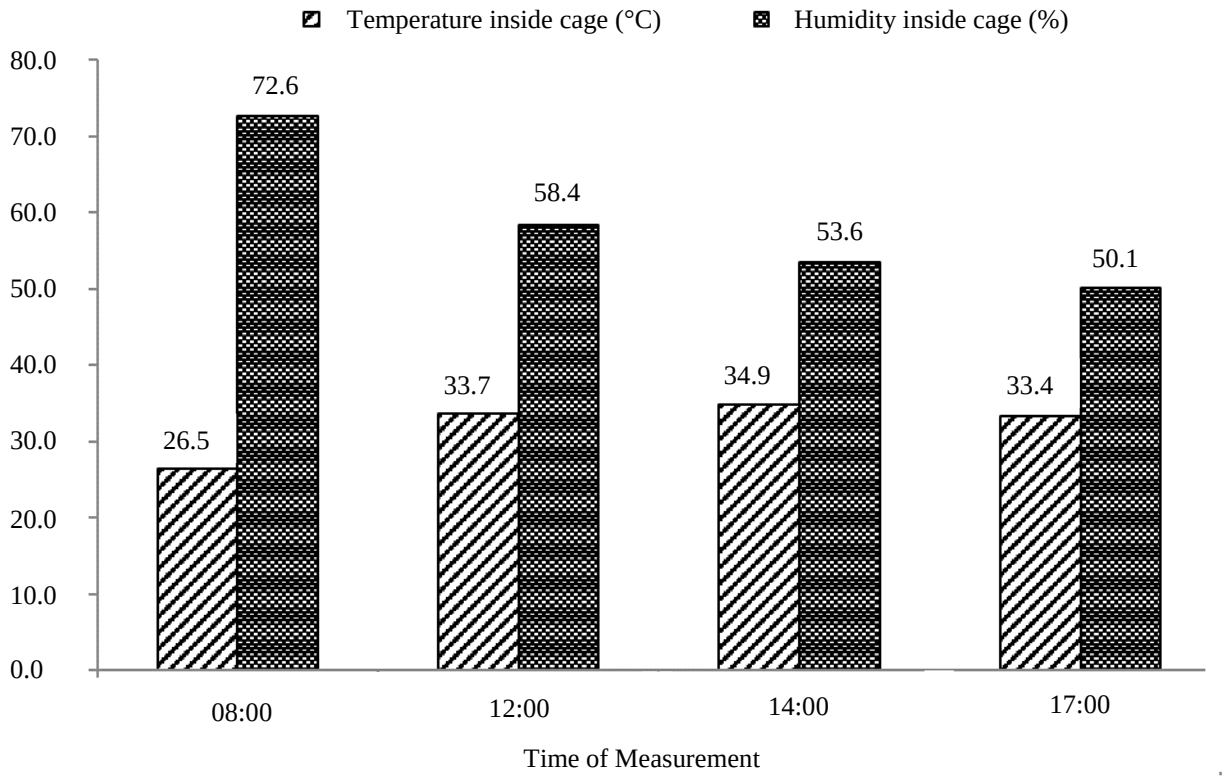

Figure 1. Average daily temperature (°C) and humidity (%) for 30 days inside cage during this study

Average yolk color, albumin height, egg weight, HU value, and eggshell thickness were shown in Table 3. Statistic test result showed that administration of *S. tetrasperma* or its combination did not affect yolk color, albumen height, egg weight, and HU value, but significantly ($P<0.01$) increased eggshell thickness. The EJ treatment tend to increase eggshell thickness. Increasing dose of turmeric and neem extract (EJ+K2 treatment) seemed did not decrease eggshell thickness. In the chicken experienced heat stress (KN treatment) may experience decrease of eggshell thickness.

Increasing of eggshell thickness of hens fed by EJ, EJ+K1, and EJK2 was suspected related to free radical forming when the hens suffering heat stress. The increasing was very high compared to that when hens in the comfort zone (24-26°C). Critical impact free radical to cell metabolism was its involvement in lipid peroxide reaction and this condition may cause damage or death of cell (Fotina et al. 2013). This was proved by administration of *S. tetrasperma* leaf extract combined with extract of turmeric rhizome and neem leaf may increase the eggshell thickness (Table 1 and 2) compared to those of EJ and KP treatments. That increasing was an effect of activity of compound therein. Bioactive compound in those extracts were suspected playing important role to decrease heat stress impact. The most compounds in those extracts were as antioxidant (Al-Harthi 2014). Result of studies showed that there were several bioactive compounds played role as antioxidant in extract of *S. tetrasperma* (Kahkonen et al. 1999; Khayyal et al. 2005; El-Wakil et al. 2015), turmeric rhizome (Gupta et al. 2010; Mohana & Fadma 2014), and neem leaf (Kumar et al. 2010).

Beside as antioxidant, several bioactive compounds in plant extract such as flavonoid compound may prevent steroid hormone activity (Zand et al. 2000). Physiology respond for body homeostatis by releasing stress hormones such as corticosteroid occurred when chicken experienced heat stress. Corticosteroid hormone disrupted body metabolism and then aggravated chicken body physiology. It was also suspected that those bioactive compounds in those *S. tetrasperma*, turmeric, and neem may disturb activity of adrenal steroidogenic enzymes and receptor sensitivity of glucocorticoid hormones (Schloms & Swart 2014; Al-Daraji 2012), so that secretion of this hormone was disrupted. This caused heat stress do not affect chicken body physiology.

Role of the extract of *S. tetrasperma*, turmeric, and neem to increase eggshell thickness was suspected through calcium and phosphor metabolism. In chicken experienced heat stress, calcium level in blood was

Table 2. Percentage of egg production, average (±SD) diet consumption, drinking water, and feed conversion ratio value of laying hen

Treatment	Egg Production (%)	Diet Consumption (g/hari/ekor)	Condumption of Drinking Water (ml/hari/ekor)	Feed Conversion ratio
KN	96.1 (346)	104.1±8.2	177.6±24.8^{ac}	2.01±0.60
KP	98.1 (353)	101.4±9.2	191.2±44.9^{a}	1.81±0.12
EJ	98.6 (355)	102.3±9.3	195.5±47.0^{a}	1.82±0.12
EJ+K1	99.4 (358)	104.7±5.5	155.5±27.1^{bc}	1.73±0.11
EJ+K2	98.3 (354)	102.3±5.8	139.9±39.3^{b}	1.83±0.16

KN = negative control treatment without anti-stress supplement
KP = positive control treatment with commercial anti-stress supplement
EJ = *S. tetrasperma* extract treatment by 1000 mg/l of drinking water dose
EJ+K1 = *S. tetrasperma* extract 1000 mg/l + turmeric 250 mg/l + neem 250 mg/l
EJ+K2 = *S. tetrasperma* extract 1000 mg/l + turmeric 500 mg/l + neem 500 mg/l of drinking water

Table 3. Average (±SD) of yolk color, albumin height, egg weight, HU value, and eggshell thickness

Treatment	Yolk Color	Albumen Height	Egg Weight (gr)	HU	Eggshell Thickness (mm)
KN	9.70±1.06	5.63±1.68	56.07±3.88	73.43±15.73	0.370±0.017^{a}
KP	9.91±1.04	6.02±1.45	55.67±4.47	77.66±9.79	0.368±0.020^{a}
EJ	10.09±1.22	6.06±1.05	56.16±4.46	78.20±6.84	0.405±0.026^{b}
EJ+K1	10.18±0.98	6.57±0.99	57.79±3.23	81.23±6.50	0.400±0.032^{b}
EJ+K2	9.75±1.14	6.13±1.64	53.25±4.10	78.04±16.64	0.399±0.021^{b}

decreased. This caused hot eggshell formation disrupted (Balnave 1998). Eggshell formation needs sufficient calcium and carbonate in the uterus. Before clacification of eggshell, calcium was not stored in the uterus, but in blood plasm in the form of calcium ion. Mineralization of calcium carbonate was done in the uterus (Ahmadi & Rahimi 2011). Radwan et al. (2008) said that several bioactive compound of plant extract may improve micro environment condition in the chicken's uterus, so that egg mineralization may occurre more perfectly and the eggshell was thicker. Activity of estrogen hormone was also suspected may increase eggshell thickness. Lukic et al. (2011) said that involvement of estrogen hormone in the calcium metabolism of eggshell was very important. Furthermore, Nakari (2005) and Noppe et al. (2008) reported that in plant extract there was phytosterol compound which may serve as steroid hormones. Sugito (2007) reported that *S. tetrasperma* plant contained steroid compound reaching 10.08%, while El-Shazly et al. (2012) has identified several phytosterols in salix plant, namely: beta-sitosterol acetate, friedelin, 3beta-friedelinol, beta-amyrin, beta-sitosterol, beta-sitosterol-O-glucoside, palmitic acid, catechol, and tremulacin. Turmeric extract contained several phytosterol (Sawant & Godghate 2013).

CONCLUSION

Administration of *S. tetrasperma* extract combined with turmeric extract and neem dissolved in drinking water for 30 days in laying hens kept in heat stress did not increase egg quality, but may increase eggshell thickness and caused decreased of water consumption.

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Lipid Profiles of Blood Serum and Fatty Acid Composition of Meat of Hybrid Duck Fed Diet Supplemented with Noni (*Morinda citrifolia*) Fruit Meal

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ABSTRAK

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Mengkudu merupakan salah satu jenis tanaman obat yang mempunyai senyawa bioaktif antioksidan yang berpotensi digunakan sebagai imbuhan pakan pada ternak unggas.Tujuan penelitian ini untuk mengetahui pengaruh penggunaan tepung buah mengkudu sebagai imbuhan pakan terhadap profil lipid serum darah dan komposisi asam lemak daging itik hibrida. Materi dalam penelitian ini adalah itik hibrida persilangan itik peking dan itik khaki campbell berumur 2 minggu sebanyak 120 ekor. Terbagi menjadi 24 unit penelitian, masing-masing berukuran 70x80x40 cm digunakan untuk 5 ekor sampai umur 56 hari pemeliharaan. Setiap unit dilengkapi dengan tempat pakan dan minum pada lantai kandang litter. Pakan penelitian digunakan pakan basal yang disusun sesuai kebutuhan nutrisi itik pedaging sesuai standar NRC (1994). Metode penelitian yang digunakan adalah uji biologis pada ternak yang dirancang dengan menggunakan Rancangan Acak Lengkap (RAL) yang terdiri dari 4 perlakuan dan 6 ulangan yaitu P0 (Pakan basal tanpa tepung mengkudu); P1 (Pakan basal + tepung mengkudu 1 %); P2 (Pakan basal + tepung mengkudu 2 %); P3 (Pakan basal + tepung mengkudu 3 %). Data yang diperoleh dianalisa ragam menggunakan Rancangan Acak Lengkap one-way ANOVA dan apabila terdapat perbedaan signifikan dilanjutkan dengan analisa beda nyata Duncan's. Hasil penelitian menunjukkan bahwa penggunaan tepung buah mengkudu sampai level 3% sebagai imbuhan pakan tidak memberikan perbedaan (P>0,05) pengaruh terhadap profil lipid serum darah dan komposisi asam lemak daging itik hibrida.

Kata Kunci: Buah Mengkudu, Fitobiotik, Asam Lemak, Lipid Darah

ABSTRACT

Kurniawan D, Widodo E, Djunaidi IH. 2015. Lipid profiles of blood serum and fatty acid composition of meat of hybrid duck fed diet supplemented with Noni (*Morinda citrifolia*) fruit meal. JITV 20(3): 200-206. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1187

Noni fruit is a medicinal plant with biological activity like antioxidant that could potentially be used as a feed additive in poultry. This research investigated the effect of noni fruit powder as feed additive on lipid profiles of blood and fatty acid compositions of meat of hybrid duck. One hundred and twenty 2-week-old hybrid ducks crossing between Peking and Khaki Campbell ducks were used in this study. They were randomly allotted to 24 experimental units. Each experimental unit was 70x80x40 cm in size and it was used for 5 ducks until they reached 56 days of age. Each unit was equipped with waterer and feeder. The ducks were raised on litter-type floor. The basal experimental diet was formulated according to the standards of National Research Council (1994). The design used for this study was experimental with 4 different treatments in 6 replications. The treatments were as follows: P0: basal feed without supplementation of noni fruit powder as control; P1: basal feed + 1 % noni fruit powder; P2: basal feed + 2 % noni fruit powder; P3: basal feed + 3 % noni fruit powder. Data were analyzed by one-way of Completely Randomized Design ANOVA and if there was any significant effect then followed by Duncan's Multiple Range Test. Result showed that using noni fruit powder as feed additive had no significant effect (P>0.05) on lipid profiles of blood and fatty acid composition of meat.

Key Words: Noni Fruit, Phytobiotic, Fatty Acid, Blood Lipid

INTRODUCTION

Increasing consumer awareness on health requires the producer to provide safe, healthty, intact and kosher food products from animal. The food products containing relatively high saturated fatty acid such as cholesterol give negative effect to the consumers. They tend to avoid the saturated fatty acid and consume low-fatty acid food products from animal. Negative effects of saturated fatty acid consumption are obesity, diabetes, hypercholesterolemia, blood vessel constriction and coronary heart diseases (Fenita et al. 2011).

It is necessary to improve food quality such as fatty acid composition in the products. One attempt is utilizing natural feed additive (phytobiotic) in poultry diet. This material is bioactive substance of medicinal plants that have been widely studied as substitution of antibiotic and may improve fatty acid composition in food products. These plants contain bioactive substances such as alkaloids, flavonoids, glycosides, saponins, and tannins which may improve health and cure diseases (Sreenivs 1999).

Noni fruit (*Morinda citrifolia*) is a medicinal plant containing a number of bioactive substances with good effects for health. About 200 phytochemicals have been identified and isolated from various parts of noni plant. Chemical composition of the noni plant is different depending on part of the noni plant. Several groups of phytochemical which have been identified from noni plant are phenol compounds organic acid, anthraquinone, anthraquinone glycosides, carotenoids, ester, flavonoid, iridoids, ketones, lactones, lignans, nucleosedes, triterpenoids, sterol and several other minor compounds. Anthraquinone is the biggest fenol compound which has been identified and isolated from noni plant. Antioxidative activity from noni plant has also widely been reported (Zin et al. 2002; Mathivanan et al. 2005; Blanco et al. 2006; Singh 2012; Kumar et al. 2014.

Nurhayati et al. (2005) reported that use of noni fruit meal up to 10% in diet had no effect on feed intake, slaughter weight, and carcass weight of broiler chicken. Fenita et al. (2011) reported that provision noni fruit water up to 75 ml/L through drinking water did not affect percentage of carcass weight, abdominal fat, cooking loss and moisture content of meat but decreased fat content by 66.52% than control. Active substance in noni fruit water such as steroid is able to block cholesterol absorption, so that it may decrease cholesterol level in blood and fat level in meat. Sujana et al. (2007) reported that use of noni fruit meal (0.1-0.4%) in diet was able to decrease cholesterol level in broiler chicken meat. Nishigaki & Waspodo (2003) said that noni fruit was able to decrease Low Density Lipoprotein (LDL) and increase High Density Lipoprotein (HDL). Adriani et al. (2014) reported that use of noni fruit juice by 0.3% and palm sugar (*Arenga pinnata*) around 0.2-0.4% through drinking water was able to decrease total cholesterol, LDL and triglyceride of blood serum of broiler chicken meat. Sally (2003) also said that use of noni fruit juice was able to increase HDL. Char (2005) said that antioxidant was able to prevent lipid (LDL) oxidation, so that oxidized LDL was not formed. Antioxidative activity on noni plant as feed additive was expected able to prevent free radical and reduce lipid oxidation on bird to produce better quality poultry products in terms of fatty acid profile.

This study was aimed to determine effect of noni meal on lipid profile of blood serum and fatty acid composition of hybrid duck meat.

MATERIALS AND METHODS

Ducks used in this study were hybrid ducks, a crossing between Peking male duck and Khaki Campbell female duck. Day old ducklings (DOD) of were obtained from a local hatchery. They were randomly allotted to 24 experimental units.

Table 1. Composition and nutrients contents of basal diet

Feed ingredients	Composition (%)
Yellow corn	56.52
Soybean meal	11.68
Fine rice bran	20.00
Fish meal	10.00
Coconut oil	1.50
Premix**	0.20
Total	100
Diet nutrient contents	
Metabolizable energy (Kcal/kg)*	3150.34
Crude protein (%)*	18.28
Crude fat (%)*	5.93
Crude fiber (%)*	4.08

(*) Analysis result of Laboratory of Nutrition and Animal Feed, Faculty of Animal Science, University of Brawijaya, Malang (2015)

(**) Premix per kg consists of:
Vit A 12.000 IU; Vit D3 2.000 IU; Vit E 8 IU; Vit K3 2 mg
Vit B1 2 mg; Vit B2 5 mg; Vit B6 05 mg; Vit B12 0.012 mg
Vit C 25 mg; Ca-D-pantothenate 6 mg; Niacin 40 mg
Cholin Chloride 10 mg; Methionine 30 mg; Lysine 30 mg
Manganese 120 mg; Iron 20 mg; Iodine 0,2 mg; Zinc 100 mg
Cobalt 0.2 mg

Lipid profile of blood serum

Method used in this study was biological test designed based on Completely Randomized Design consisting of 4 treatments and 6 replications as follows:
P0: Basal diet without noni fruit
P1: P0 + 1% noni fruit meal
P2: P0 + 2% noni fruit meal
P3: P0 + 3% noni fruit meal

Noni fruit used was yellow ripe noni. Noni fruit meal production was started by cleaning the fruit by flowing water. The clean fruits were cut crosssection by 2-3 cm in thick and dried over in oven 45°C for 2-3 days. Dried noni fruits were ground finely and sifted using 75 meshes (0.75 mm). The hybrid ducks were kept in litter cage by 70 cm X 80 cm X 40 cm. Each cage was filled up by 5 ducklings and grouped by treatments. Each cage was equipped with waterer and feeder. They were fed by the allocated treatments ad libitum in the morning (07.00 am) and afternoon (03:00 pm). Drinking water was given ad libitum. Treatments application and data collection were conducted for 6 weeks. Treatment diet was mixed according to basal diet composition based on broiler duck nutrient requirement in the NRC (1994). Basal diet composition and nutrient contents used in this study were presented in Table 1.

Blood sample collection was carried out on 1 duck from each replication at the end of study (after 6 weeks). Blood was taken from wing vena by 3 ml syringe and then was placed in the sample tube. Blood sample was placed in Styrofoam box containing ice cubes and transported to the laboratory. In the laboratory, blood sample was placed in 4°C refrigerator for 12 hours before it was centrifuged by 3500 rpm for 10 minutes. Supernatant, in the form of serum was taken using sterile pipette and placed in eppendorf tube for further analysis. Analysis of lipid profile of blood serum consisted of triglyceride level, total cholesterol, Low Density Lipoprotein (LDL), and High Density Lipoprotein (HDL) using CHOD-PAP method (Cholesterol Oxidase Phenylperoxidase Amino Phenozonphenol) based on DSI (2005).

Fatty acid composition

Meat sample collection was done by randomly slaughtering the ducks in the end of maintenance (8 weeks) from each replication in every treatment. Meat samples used were from chest and thigh. Testing procedure of meat fatty acid composition was according to AACC (1983). Testing steps were consisting of soxhlet extraction to produce fat in the form of oil. Methylation was done by refluxing 0.02 g fat over a water bath (80°C) with 5 ml NaOH in methanol and 5 ml bourtiflourid-metanol respectively for 20 minutes, and than were removed and cooled. The next step was addition of 2 ml saturated NaCl and 5 ml hexane and homogenized. Hexane lining was pipetted and put into eppendorf tube. Chromatography gas was injected into 2-5 µL sample. Fatty acid identification using the chromatography gas with column temperature at 200°C, initial temperature at 150°C, final temperature at 180°C, limit pressure at 3000 psi, N2 mobile phase, stationer phase of diethylene glycol succinate (DEGS) powder, detector FID 250°C, column length 40 m and diameter in column 1.2 mm. Standards of fatty acid used were capric acid (C10: 0), lauric acid (C12: 0), myristic acid (C14: 0), palmitic (C16: 0), stearic (C18: 0), oleic (C18: 1), linoleic (C18: 2) and linolenic acid (C18: 3). Calculation of fatty acid referred to Dewi (2009) as follow:

The number of fatty acid A (mg/g):

$$= \frac{\textit{Weight SI on sample (mg)}}{\textit{Weight of sample (g)}} \text{ X RF X } \frac{\textit{Area of sample fatty acid}}{\textit{Area standard}}$$

RF fatty acid A:

$$= \frac{\textit{SI area}}{\textit{Weight of sample (g)}} \text{ X } \frac{\textit{Fatty acid consentration of standard}}{\textit{Fatty acid area A of standard}}$$

where:
SI = Internal standard C17 (margarat acid)
RF = Factor response

Statistics analysis

Data obtained were tabulated using excel and analyzed using Completely Random Design if there was significant or very significant effect then followed by Duncan's Multiple Range Test with following mathematical model:

$$Yij = \mu + \tau i + \varepsilon ij$$

where:
Yij = Observation result of change of noni fruit meal use i_{th} with j-replication
µ = Average observation
τí = Effect of i-treatment
εij = Effect of i-trial error and j-replication
i = 1, 2, 3, and 4
j = 1, 2, 3, 4, 5, and 6

RESULTS AND DISCUSSION

Lipid profile of blood serum

Effects of noni fruit meal to lipid profile of blood serum is presented in Table 2. Noni fruit meal used as feed additive had no significant effect ($P<0.05$) on triglyceride, total cholesterol, HDL and LDL. Administration of noni fruit meal up to 3% in diet was not able to decrease triglyceride level and total cholesterol of duck's blood serum. This result is not in accordance with Adriani et al. (2014) who reported that there was a decrease of triglyceride and total cholesterol of broiler chicken's blood serum by administrating noni fruit juice through the drinking water. Coumarin compound increases secretion of biliary acid and oxide nitrite compound, which will secrete cholesterol in the blood through feces. Beta-carotene and flavonoid also have role in decrease of cholesterol level. Sujana et al. (2007) reported that cholesterol content of broiler chicken meat experienced a decrease by administrating diet containing of noni fruit meal. Fat level was also decreased in broiler chicken meat by administrating noni fruit water as reported by Fenita et al. (2011).

Active compound in noni fruit is one of causes in the decrease of level of triglyceride and cholesterol in the blood serum. Administrating noni fruit meal up to 3% in diet has not been able to decrease level of triglyceride and cholesterol of hybrid duck's blood serum. This shows that active compound in noni fruit meal is unable to reduce lipid oxidation and prevent cholesterol formation.

Phytosterol is plant fat which may not be absorbed by digestive track. The phytosterol may increase partly of biliary acid, so that availability of biliary acid is dwindling. Cholesterol from diet may not be absorbed by intestinal wall because it reacts with the biliary acid (Anwar & Piliang 1992). Phenol compound, especially flavonoid, may prevent intestinal michellium formation, a place of biliary acid absorption, so that cholesterol may not be absorbed by digestive track and then immediately removed through feces (Kusnadi 2004).

This research showed that administrating noni fruit meal up to 3% was unable to decrease LDL level or increase HDL level of hybrid duck's blood serum. This shows that active compound in noni fruit meal was unable to prevent lipid oxidation. This result was not in line with research result of Adriani et al. (2014) who said that administration of noni fruit juice and sugar palm in the drinking water of broiler chicken may decrease total cholesterol, LDL, and triglyceride of blood serum. As reported by Fondevilla et al. (2010) that administration of noni fruit meal to mouse decreased level of cholesterol, triglyceride, LDL and HDL in blood serum. Xeronim compound decreases level of cholesterol in blood because it stimulates insulin hormone formation. Insulin hormone is able to increase the number of receptor of Very Low Density Lipoprotein (VLDL), where this compound will bring triglyceride-rich particles in the bloodstream from liver to body cell.

Composition of meat fatty acid

Composition of fatty acid of duck meat supplemented by noni fruit meal is presented in Table 3. Results showed that duck meat contained 11 fatty acids belonging to 8 saturated fatty acids namely: Butyric (C4), caproic (C6), caproate (C8), caprate (10), lauric (C12), myristic (C14), Palmitate (C16-0) and stearic (C18-0) and 3 unsaturated fatty acids namely: Oleic (C18-1), linoleic (C18-2), and linoleic (18-3). Control treatment (without noni fruit meal) had fewer fatty acids than other treatments. There were several undetected sort-chain saturated fatty acids in the control treatment such as Butyric (C4), caproic (C6), caproate (C8) and caproate (10).

Duck meat has higher unsaturated fatty acid content compared to saturated fatty acid. This is in line with Aronal et al (2012) who said that Muscovy, Peking duck and wild duck had higher unsaturated fatty acid

Table 2. Lipid profile of blood serum of hybrid duck supplemented by noni fruit meal in diet

Parameter	PO	P1	P2	P3
Cholesterol (mg/dL)	203.33±43.82	197.00±60.11	186.33±65.06	192.00±58.03
Triglyceride (mg/dL)	155.33±43.82	191.33±11.40	183.00±11.45	172.33±91.78
HDL (mg/dL)	111.40±43.82	91.63±21.63	92.77±28.76	94.10±29.71
LDL (mg/dL)	60.87±43.82	67.10±21.61	56.97±23.69	56.77±22.57

P0 = Basal diet without noni fruit meal (control)
P1 = Basal diet + 1% noni fruit meal
P2 = Basal diet + 2% noni fruit meal
P3 = Basal diet + 3% noni fruit meal

Table 3. Composition of fatty acid (mg/g) of hybrid duck meat supplemented by noni fruit meal in diet

Fatty acid	Treatment			
	P0	P1	P2	P3
Butyrate (C4)	-	-	0.01±0.00	0.02±0.00
Caproate (C6)	-	0.01±0.00	0.01±0.00	0.01±0.00
Caprilate (C8)	-	0.01±0.00	0.01±0.00	
Caprate (10)	-	-	0.01±0.00	-
Laurate (C12)	0.04±0.00	0.01±0.00	0.02±0.00	0.01±0.00
Myristate (C14)	0.09±0.01	0.09±0.01	0.11±0.02	0.09±0.00
Palmitate (C16-0)	3.85±0.41	4.02±0.68	4.60±0.57	4.05±0.29
Stearate (C18-0)	$1.32±0.12^{a}$	$1.48±0.25^{ab}$	$1.89±0.17^{c}$	$1.75±0.16^{bc}$
Total ALJ	5.30±0.49	5.61±0.95	6.65±0.66	5.94±0.44
Oleate (C18-1)	7.04±0.83	7.03±1.36	7.95±0.94	7.16±0.77
Linoletae (C18-2)	2.32±0.28	2.32±0.36	2.85±0.33	2.54±0.16
Linoleate (C18-3	0.09±0.01	0.08±0.01	0.10±0.02	0.07±0.01
Total ALTJ	9.45±1.15	9.43±1.70	10.90±1.76	9.77±0.91
Ratio ALTJ/ALJ	1.78±0.14	1.67±0.16	1.49±0.18	1.64±0.04

Different superscripts in the same row show significant (P<0.05) differences - (undetected)
ALJ = Saturated fatty acid
ALTJ = Unsaturated fatty acid
P0 = Basal diet without noni fruit meal (control)
P1 = Basal diet + 1% noni fruit meal
P2 = Basal diet + 2% noni fruit meal
P3 = Basal diet + 3% noni fruit meal

composition than saturated fatty acid. Pikul et al (1996) said that bird had better fatty acid content than other animals because they had 60-70% polyunsaturated fatty acid (PUFA) and 45-50% monounsaturated fatty acid (MUFA). Fatty acid profile of bird is affected by diet content fed to bird. Bird may synthesize saturated fatty acid and the MUFA in the diet, except fat. Cho et al. (2005) said that fatty acid composition in meat and fat tissue of animal was affected by several factors such as: species and breed, sex, and diet composition.

In this study, administration of noni fruit meal in diet did not affect (P>0.05) overall fatty acid composition of duck meat, saturated fatty acid, and ratio of saturated fatty acid and unsaturated fatty acid (STF/UFA ratio), except stearat (C18-0) which showed significant effect (P<0.05)

Based on result of further analysis of DMRT, administration of noni fruit meal (2 and 3%) showed higher stearate acid than the control. This result shows that administration of noni fruit meal up to 3% in diet was unable to change composition of meat fatty acid. Active compound in the noni fruit meal was unable to prevent lipid oxidation, so that duck meat fatty acid composition was not different from the control. Different result was reported by Rukmiasih et al. (2011) who said that composition of unsaturated fatty acid of duck meat increased by administration of *beluntas* plant (*Pluchea indica* L) combined with vitamin E compared with control (without vitamin E). This shows that antioxidant activity of natural material such as the *beluntas* plant and vitamin E was more effective in protecting the unsaturated fatty acid from excessive lipid oxidation. Unsaturated fatty acid is a compound that is susceptible to auto-oxidation. Lipid oxidation is the main cause of bird products damage. Antioxidant compound is a component that may retard and prevent lipid oxidation by free radical. Alloui et al. (2014) said that antioxidant in several herbal plants was able to protect lipid in diet from oxidation, so that the products from animal became more stable to the oxidation. Purba et al. (2010) said that composition of fatty acid of boiled duck meat supplemented by antioxidant showed higher total composition of unsaturated fatty acid than the total of saturated fatty acid.

Content of stearate acid was increased by administration of noni fruit meal. This was caused by fatty acid content of noni seed oil in the noni fruit meal. Timotius (2003) said that noni seed consisting of fewer oil than other vegetable oil-source plants. Noni seed oil consists of many unsaturated fatty acid such as linoleate

and oleat acids about 75%. The rest, about 25% was saturated fatty acids such as palmitate and stearate acids. Composition of this fatty acid is also related to bioactive compound in the noni seed. Singh (2012) said that in noni fruit, there was an antaquinine substance which is an acid material and closely related to cathechol (acid). Noni meal may affect pH of digestive track to become more acidic. Acidic condition will cause activity of lipase enzyme to be restricted, so that it causes reducing of fat digestion and fewer body fat forming. Furthermore, it is said that noni meal has direct role in blood vessel and may neutralize fat which will be transported to meat forming.

CONCLUSION

Administration of noni fruit meal up to 3% in diet has not been able to change lipid profile of blood serum and composition of fatty acid of hybrid duck. Stearat acid experienced an increase by administration of noni fruit meal.

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Production and Quality of *Murdannia bracteata* Biomass as Impact of Magnesium Foliar Fertilizer

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ABSTRAK

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Murdannia bracteata adalah salah satu hijauan pakan yang berpotensi sebagai pakan ternak ruminansia dan belum banyak diteliti. Hijauan ini mengandung mineral yang tinggi dan berpotensi sebagai pakan fungsional. Tujuan dari penelitian ini adalah untuk mengevaluasi pertumbuhan, produktivias serta kadar klorofil dan mineral *Murdannia bracteata* yang dipupuk dengan pupuk daun magnesium. Penelitian ini menggunakan rancangan acak lengkap dengan 5 perlakuan dan 4 ulangan. Perlakuan dosis magnesium yang diberikan adalah 0 ppm, 2000 ppm, 4000 ppm, 8000 ppm, 12000 ppm. Penelitian dilakukan di rumah kaca laboratorium lapang agrostologi, Fakultas Peternakan, IPB. Hasil penelitian menunjukkan bahwa penambahan dosis pupuk magnesium tidak memberikan pengaruh yang nyata (P>0,05). terhadap pertumbuhan, produktivitas, dan kadar klorofil. Peningkatan dosis magnesium menyebabkan turunnya kadar kalsium, kalium, dan zink (P<0,05), namun tidak pada phosphor dan natrium (P>0,05). Kesimpulannya, pemberian pupuk daun magnesium hingga dosis 12000 ppm tidak memberikan respon negatif terhadap pertumbuhan, produktivitas, serta kualitas tanaman. Peningkatan dosis pupuk magnesium mengakibatkan penurunan beberapa kandungan mineral dalam tanaman, khususnya kalsium, kalium dan zink.

Kata Kunci: *Murdannia bracteata*, Magnesium, Produktivitas, Kualitas

ABSTRACT

Rahmawati T, Abdullah L, Prihantoro I. 2015. Production and quality of *Murdannia bracteata* biomass as impact of magnesium foliar fertilizer. JITV 20(3): 207-213. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1188

Murdannia bracteata is one of potential forages for ruminant that has not been studied yet. This forage contents high mineral and it can be as fungtional feed. The aim of this study was to evaluate the growth and productivity, chlorophyll and minerals content of *Murdannia bracteata* caused by magnesium foliar application. The experiment was arranged in randomized complete design with five treatments and 4 replications. The application of magnesium level was arranged into: 0 ppm, 2000 ppm, 4000 ppm, 8000 ppm, and 12000 ppm. The study was conducted in a greenhouse, field laboratory of Agrostology, Faculty of Animal Science, Bogor Agricultural University. The result showed that growth, productivity, and chlorophyll content were not significantly (P>0.05) affected by increasing magnesium level. Increasing magnesium dossage resulted in decreasing calsium, potassium, and zink content (P<0.05), but not on phosphor and sodium (P>0.05). In conclusion, increasing magnesium dossage up to 12000 ppm did not significantly affect growth, productivity, and chlorophyll content. However, increasing magnesium level decreased calsium, potassium, and zink content.

Key Words: *Murdannia bracteata*, Magnesium, Productivity, Quality

INTRODUCTION

Murdannia bracteata is one of forage has not been widely studied. This potential forage as ruminant's functional feed included into class Commelinales, family Commelinanceae, genus Murdannia and species *M. bracteata* (C.B. Clarke) Kuntze ex D.Y. It has advantage as crude fiber source may be used as anti-inflamatory and high mineral content (Wang et al. 2007, Rahmawati 2014). Administration of organic fertilizer by 10 ton ha^{-1} and 37% shading affect the height of level of calcium (Ca), magnesium (Mg), calium (K), and zink (Zn). Content of those Ca, Mg, K, and Zn is 16847 ppm, 4566 ppm, 29323 ppm, dan 44 ppm, respectively.

Those minerals content were higher compared to other forages such as *Brachiaria humidicola* and *Indigofera sp*. Minerals contained in this forage is potentian as organic magencium source for animal.

Magnesium (Mg) is macro mineral needed by ruminants. Mg is mineral which is closely related to calcium. About 70% magnesium is found in bone. This mineral plays a role as enzyme activator and key of cellular biochemist process (McDonald et al. 2010). Administration of 10% DM of *M. bracteata* as magnesium source in beef cattle's diet may increase

VFA production, DM and organic material digestibility, and microbe protein synthesis (Rais 2015).

Magnesium has important role for the crop, among other as the main element of chlorophyll forming, playing role in crop metabolism such as photosynthesis and charbohydrate forming (Gerendàs & Führs 2013), playing role in nitrogen metabolism, enzyme activator, and nucleate acid synthesis (Salama et al. 2014). Magnesium deficiency in crop was characterized by chlorosis between the leaf bone, but the leaf was still green (Chalimah & Sulaiman 2015). Therefore, magnesium must be available in sufficient number. The main source of mineral on crops is origine from soil. There are several soils with low nutrient content, such as darmaga latosol soil. Magnesium content in the darmaga latosol soil was only 0.06% (Utami 2015). Therefore, it needed magnesium fertilization. Magnesium fertilization was given by several ways. One of those ways is through the leave known as foliar fertilization. Foliar fertilization is fertilizer diluted by water and sprayed on the leaves (Hanadyo et al. 2013). That liquid fertilizer entered the leaves by penetration process to cuticle tissue through stomata pathway (Aghtape et al. 2011). This fertilizer has advantage. Nutrient given directly may be absorbed through the stomata pathway. Sprayed fertilizing should not in large amount.

The *M. bracteata* has high magnesium content. In this study, administration of foliar fertilizer was carried out to determine capability of that *M. bacterata* in increasing magnesium content without disturbing the growth, productivity, and its quality planted on lasotol dramaga soil.

MATERIALS AND METHODS

This research was carried out in a greenhouse of Field Laboratorium of Agrostology, Laboratorium of Animal Nutrition, Faculty of Animal Science, Bogor Agriculture University. Study was carried out for 4 months started from Februari 2015 until Mei 2015. It consisted of planting the *Murdannia bracteata* in polybag and measurement of chlorophyll and several minerals level.

Equipments used in this study were digital weigher scale 1 gram, gauge, polybag with diameter 40 cm, oven 60^o C Swallow LTE.Scientific LTF K11755, oven 105^o C, and minerals analizing tools. Materials used was seed of *Murdannia braceata*, organic fertilizer 10 ton/ha (commercial fertilizer), lime, $MgSO_4.7H_2O$, commercial NPK and latosol dramaga soil.

The *Murdannia bracteata* was maintained for 2 months fertilized everyweek started in third week. Fertilization was done by spraying 2-3 ml magnesium liquid. Administration of organic fertilizer lime, and NPK was done once at the beginning of planting. Watering and pest weeding were done everyday. The *Murdannia bracteata* was harvested after 8 weeks with sparating stems, leaves, roots, and flowers for weighing. Sampels were putted into oven 60^{o}C, oven 105^{o}C, and furnace to determine level of dry and organic materials. One leaf in every experimental unit was sparated to be anallized its chlorophyll content. Those leaf samples were taken randomly from every crop. Samples preparation was done referring to method of Sims & Gamon (2002) and it was then seen its absorbance using spectrophotometer with wave length by 537, 663, 647 nm.

Anthocyanin = 0.08173*A537 – 0.00697*A647 – 0.002228*A663

Chlorophyll a (*Chl a)* = 0.01373*A663 – 0.00089*A537- 0.003046*A647

Chlorophyll b (*Chl b)* = 0.02405*A647 – 0.004305*A537 – 0,005507*A663

$$\text{Caroten} = \frac{(A470 - (17.1 * (Chl\ a + Chl\ b) - 9.479 * antosianin))}{119.26}$$

Total Chorophyll = *Chl a + Chl b*

Every experimental unit of leaf samples which have been analized for organic materials was analized content of Ca, P, Mg, K, Na and Zn using wet incorporation method Reitz et al. (1960). Concentration of those minerals level were measured using atom absorption spectrophotometer (AAS), and the mineral P was measured using spectrophotometer (UV Visible) with wavelengath by 660 nm.

Levels of magnesium given were 0 ppm, 2000 ppm, 4000 ppm, 8000 ppm, and 12000 ppm. This study was used completely randomized design with 5 treatments and 4 replications. Data obtained were tested by Analysis of Variance (ANOVA) using SPSS 16 software. If there was a significant difference, orthogonal polynomial was done (Matjik & Sumertajaya 2006).

RESULT AND DISCUSSION

Murdannia bracteata growth

Plant growth consists of 2 phases, namely vegetative and generative phase. Vegetative phase consist of development of root, leaf, and stem. Generative phase is a reproductive phase consisting of forming and development of flower and bud. Plant growth is metabolic result of life cells requiring carbohydrate and may be measured quantitatively. The *Murdannia bracteata* growth by adding magneciun in different level was presented in Table 1 and 2.

Administration of magnesium foliar fertilizer with different level did not significantly (P>0.05) affect the vegetative growth of *Murdannia bracteata*. Administration of magnesium foliar fertilizer up to 12000 ppm showed the same vegetative growth effect to the control.

This shows that administration of magnesium in that level did not affect the *Murdannia bracteata* growth. Growth disruption due to toxicity of magnesium was marked by disruption increase of plant height growth and leaf wide growth due to an antagonism between the magnesium and calcium (Fitriyatno et al. 2012). Leaf wide did not response significantly. This showed that the leaf may do good photosynthesis unmolested by administration of magnesium foliar fertilizer.

Administration of magnesium foliar fertilizer with different level did not significantly (P>0.05) affect generative growth of the *Murdiannia bracteata*. Administration of that fertilizer in 12000 ppm did not affect generative growth of the *Murdiannia bracteata*. This shows that the magnesium foliar fertilizer administration up to that level may still be tolerated by the *M. bracteata*. Crops that are less tolerant will show negative response, such as decrease of generative growth with increase of magnesium level. However, toxicity of magnesium in crops is rarely happening. Excess of magnesium in crops will be squestered in vacuola (Tang et al. 2015).

Productivity of *Murdannia bracteata*

Productivity of the *Murdannia bracteata* was divided into fresh weight and dry weight. Fresh weight and dry weight are one of growth indicators of crop. Dry weight is an indicator of the number of organic material successfully synthesized by crops from anorganic matterials.

Average fresh and dry weight of the *Murdannia bracteata* were presented in Table 3. Administration of magnesium foliar fertilizer in different level did not significantly (P>0.05) affect biomass production, and vegetative and generative growth rate of the *M. bracteata*, so that productivity of the *M. bracteata* was also not significantly different. Crops that are not tolerant to magnesium stress will show negative response, such as decrease of its produvtivity because of disruption of carbohydrate metabolism due to decrease of Zn content in the crops.

Increase of magnesium content in crops may decrease Zn content and then preventing carbonic anhydrase enzyme, so that the carbohydrate is concentred in the crown (Barker & Eaton 2015). Non-edible fresh and dry weight in this study was not affected by magnesium fertilizing. This shows that carbohydrate metabolism in this crop was not disturbed by high of magnesium foliar fertilizer level administered. Carbohydrate metabolism in the root and

Table 1. Vegetative growth of *M. bracteata* as an impact of magnesium foliar fertilizer administration in different level

Level of magnesium (ppm)	Increase of crop length (cm/week)	Increase of the number of leaves (cm/week)	Wide of leaves (cm)
0	2.72±0.49	16.56±2.61	1.89±0.10
2000	3.30±0.39	19.09±2.84	1.98±0.10
4000	3.05±0.34	20.79±3.43	1.95±0.08
8000	2.87±0.38	19.31±4.12	1.94±0.05
12000	3.01±0.15	22.19±2.01	1.97±0.07

Table 2. Generative growth of *M. bracteata* as an impact of magnesium foliar fertilizer administration in different level

Level of magnesium (ppm)	Increase of the number of flower (cm/week)	Increase of the number of bud (cm/week)	Stolon length (cm)
0	3.25±2.85	2.72±0.62	66.88±37.72
2000	5.38±2.02	2.66±0.48	88.00±22.38
4000	4.97±2.62	3.44±0.24	71.00±2.83
8000	2.88±0.47	2.94±1.04	89.13±9.78
12000	2.97±0.41	3.84±0.90	108.38±29.16

crown was running normally. Inhibition of the carbohydrate metabolism will disrupt metabolism of crop cells, decreasing crop growth, and then will affect the productivity. This is suspected due to administration of organic fertilizer as basic fertilizer by 10 ton ha^{-1} in this study has fulfilled nutrient balance required by the crop. Organic fertilizer has an important role in improving physical, chemical, and biological characteristics of soil, so that it may increase land productivity and fertilizer efficiency (Supartha et al 2012). In a provious study, administration of organic fertilizer by 10 ton ha^{-1} showed the best productivity of the *M. bracteata* (Rahmawati 2014). Table 3 shows that decrease pattern of dry matter. By administration of magnesium fertilizer up to 8000 ppm, the crop was still able to maintain dry weight by 11-12%, but in administration of magnesium fertilizer decreased the dry weight into 8%. Administration of magnesium fertilizer up to 12000 ppm increased magnesium content in crop and was suspected increasing water content level. Magnesium is an ion that has 2 cations. Those cations have high affinity to various ligands and are surrounded by 2 water molecules, namely: inner layer and outer layer. The inner layer consists of 6 water molecuIs forming magnesium complex in the form of octahedral [Mg(H2O)6] 2+. Outer layer consists of 12 water molecuIs (Grzebisz 2015).

Quality of *Murdannia bracteata*

Quality of *M. braceata* based on mineral content, mineral absorbtion, and chlorophyll influenced by administration of magnesium in different level was presented in Figure 1, Table 4, and Table 5. Analysis of variance result of mineral content of the *M. bracteata* with magnesium administration in different level showed significant ($P<0.05$) result to Ca, Mg, K, and Zn, but it was no significant ($P>0.05$) effect to P and Na. Increasing level of magnesium affected increase of Mg and decrease Ca, C, and Zn. This shows that spraying of foliar fertilizer given direct effect against nutrient content. Fertilizing through leaves is one of alternatives fertilizing has quicker reaction and more efficient with the relatively few use of fertilizer (Portu et al. 2015).

Mineral has synergic and antagonic characteristics between one and others. Increasing of one mineral in crops will decrease the other minerals content. Addition of magnesium will increase magnesium content and decreasing Ca, K, and Zn. This shows that magnesium sprayed through leaves was penetred into leaves, so that may increase magnesium content and decreasing the other minerals content (Figure 1). In the same figure, it is also showed that there is about the same antagonistic pattern between Mg, K, and Zn. The two graphycs show that in spraying of foliar leave in 2000 and 4000 ppm there was magnesium saturation, so that in those two levels, magnesium level is not different. This caused content of K and Zn in those levels did not significantly decrease. The same thing was in spraying in 8000 and 12000 ppm.

Mineral in crop is absorbed in the form of ion. High concentration of magnesium may cause deficiency of other substantial cations. Magnesium toxicity symptom was highly related to decrease of Ca and K content (Merhaut 2007). However it showed toxicity symptom, the growth and productivity of the *M. bracteata* did not show negative response. Crop may maintain anion-cation balance if it is added by some minerals expect kalium (Mengel 2007). This shows that addition of Mg^{2+} to crop replaces other cations. It causes decrease of cation content of calcium, kalium, and zinc. Magnesium spraying to crop increased magnesium content. High magnesium content out of cell caused stress censor of magnesium in the active plasm membrane. Stress censor of the magnesium is calcineurin B-like (CBL) 2 and 3. CBL-2 and CBL-3 will interact with calcineurin protein kinase (CPIK) to manage magnesium absorption into vacuola. There are other censors around the vacuola, such as CBL-10 for

Table 3. Production of fresh and dry biomass of *M. bracteata* based on level of magnesium fertilizing

Level of magnesium (ppm)	Fresh weight (g)		Dry weight (g)	
	Edible	Non-edible	Edible	Non-edible
0	136.5±34.7	32.6±16.6	14.2±6.8	4.7±3.1
2000	142.3±26.3	35.1±11.5	13.23±8.7	8.2±3.2
4000	161.1±30.8	46.5±16.6	16.5±7.0	5.4±3.7
8000	120.3±30.4	27.4±16.7	13.1±1.2	4.1±3.9
12000	158.1±13.8	43.0±12.8	9.9±1.7	5.8±4.1

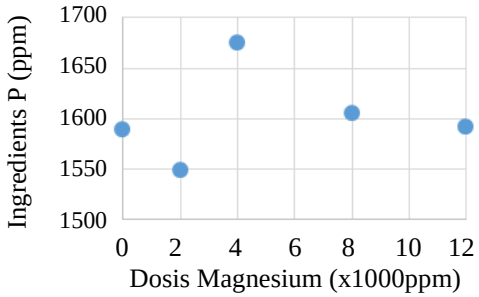

Figure 1a

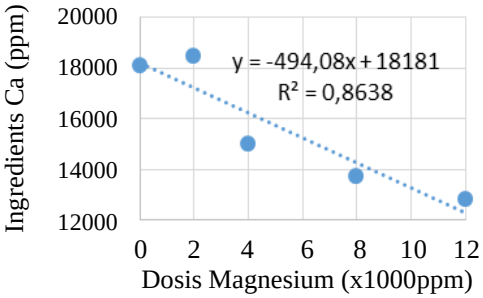

Figure 1b

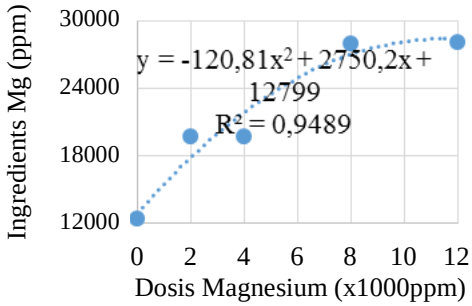

Figure 1c

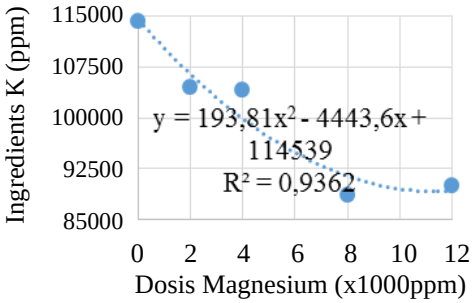

Figure 1d

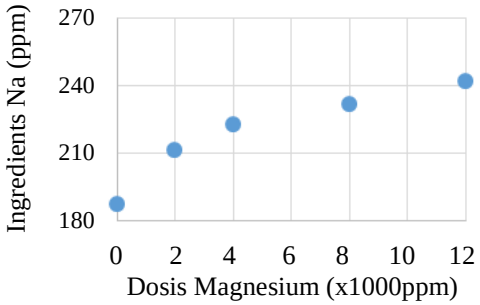

Figure 1e

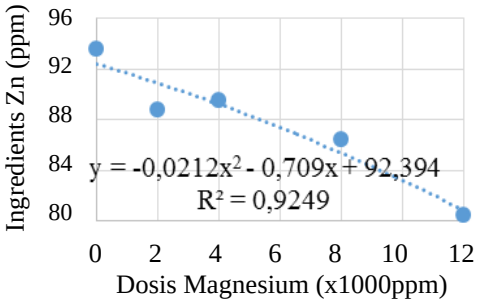

Figure 1f

Figure 1. Administration of magnesium foliar fertilizer with minerals content of *M. bracteata* (1a: Phosphor, 1b: Calcium, 1c: Magnesium, 1d: Kalium, 1e: Natrium, 1f: Zink)

natrium and CBL 1 & CBL-9 for kalium. When the natrium content is increase, CBL-10 will give signal to plasm membrane to absorb natrium content excess and it is stored in vacuola and the CIPKs24 will give signal to decrease natrium content in the plasm membrane. When kalium concentration in cell membrane is decrease, censor CLB-1 & CLB 9 will give signal to plasm membrane to increase absorption of kalium in the cell membrane (Gao et al. 2015a). Vacuola was an organ which has several functions, such as in saving nutrition and metabolic, degradation of protein, and plant defense (Gao et al. 2015b).

Table 4. Macro mineral absorption of *M. bracteata* leaves as effext of magnesium foliar fertilizer application in different level

Level of magnesium (ppm)	P	Ca	Mg	K	Na	Zn
	---------- mg crop^{-1}----------					
0	16.8±8.7	182.8±92.8	121.8±53.9	1128.8±468.7	1.8±0.9	0.9±0.4
2000	11.1±7.6	159.9±145.9	171.5±143.4	869.0±743.9	1.9±1.4	0.7±0.6
4000	20.0±11.6	136.1±122.8	218.1±133.8	931.3±477.4	2.4±0.9	1.0±0.5
8000	13.0±2.9	122.8±74.5	235.1±83.7	752.3±289.6	2.0±0.8	0.7±0.3
12000	9.5±4.3	73.7±15.4	162.5±32.5	533.7±176.1	1.4±0.2	0.5±0.2

Table 5. Chlorophyll level of *M. bracteata* as effect of magnesium foliar fertilizer application in different level

Level of magnesium (ppm)	Chlorophyll A	Chlorophyll B	Antocianine	Caroten
	---------- mg g^{-1} ----------			
0	0.53±0.14	0.22±0.06	0.02±0.01	0.16±0.04
2000	0.47±0.09	0.20±0.04	0.03±0.01	0.14±0.02
4000	0.48±0.10	0.21±0.05	0.03±0.01	0.14±0.03
8000	0.58±0.06	0.25±0.02	0.02±0.00	0.18±0.02
12000	0.56±0.04	0.24±0.03	0.03±0.01	0.17±0.01

Mineral level of *M. bracteata* was including to high grade refered to McDonald et al (2010) compared with mineral level of grass in temperate area, even mineral level of kalium and phosphor of *Murdannia bracteta* was lower than in *Indigofera sp.* (Herdiawan 2013). High mineral level in this crop is potential as an organic mineral source for slow release animal.

Result of analysis of variance showed that addition of magnesium did not significantly (P>0.05) affect absorbtion of mineral. This showed that the amount of mineral absorbed in different level showed the same result. It was suspected due to nutrient in the soil meets nutrient requirement for optimal grow. This mineral absorbtion also showed that minerals used for metabolism in crop were enough to fulfill vegetative and generative growth.

Chlorophyll is green substance which closely related to absorption of light and process of photosynthesis. Chlorophyll is divided into chlorophyll A ($C_{55}H_{72}O_5N_4Mg$) with dark green color and chlorophyll B ($C_{55}H_{70}O_5N_4Mg$) with light green color (Wihermanto & Handayani 2011). Magnesium is a core mineral in the chlorophyll. Magnesium atom, which was photosynthesis process determinant, was very decisiving productivity process of crops (Gransee & Fuhrs 2013). Addition of magnesium foliar fertilizer in different level did not significantly (P>0.05) affect content of chlorophyll of the *M. bracteata*.

This is suspected because of forming of chlorophyll of *M. bracteata* was optimal, so that when the magnesium foliar fertilizer was added up to 12000 ppm, it did not give significant affect. Besides, the amount of magnesium absorption in every crop did not show significant affect, so that the magnesium added will directly be stored in vacuola. The amount of magnesium absorbed did not increase, therefore chlorophyll level also did not increase indirectly. Magnesium used by crops for photosyntesis process was about 20% of total magnesium in the crop, whereas the rest of 80% was left mobile in the crop (Marschner 2012). Addition of high magnesium did not cause toxivity to the *M. bracteata*.

CONCLUSION

Addition of magnesium foliar fertilizer up to 12000 ppm increased magnesium content and did not give negative effect to growth, productiovity, and quality of crop. Increasing of magnesium level affected decrase of several minerals content (Ca, C, and Zn) in crop.

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Pathological Changes of Suspected Tetrachloro dibenzo-ρ-dioxins/Tetrachloro dibenzofurans Toxication in Beef Cattle

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ABSTRAK

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Kontaminasi tetrachloro dibenzo-ρ-dioxins (TCDDs) dan tetra chlorinated dibenzofurans (TCDFs) dapat mempengaruhi kesehatan masyarakat dan hewan seperti kanker, gangguan reproduksi, keracunan kulit dan gangguan neuorologi. Tujuan penelitian ini adalah mempelajari perubahan patologis kontaminasi TCDD/Fs dalam pakan pada berbagai jaringan tubuh sapi potong yang mana TCDD/Fs dideteksi dengan GC MS/MS. Hasil penelitian menunjukkan bahwa POPs (seperti DDT, heptakhlor, aldrin, dieldrin dan endrin) sebagai pemicu pembentukan dioksin terdeteksi pada semua sampel kecuali air minum ternak. Konsentrasi total OC dalam tanah antara tt – 42,73 μg/kg, rumput (3,30 – 27,66 μg/kg), air sumur (0,82 – 1,00 μg/kg), konsentrat (3,90 μg/kg), serum (tt – 13,08 μg/kg) dan daging (tt – 100,72 μg/kg). Lebih lanjut nilai TEQ residu TCDDs/Fs pada daging sapi di Yogyakarta adalah 4.496,66 - 20.642,40 pg/g dan 717.13 pg/g (daging sapi) dan 0.037 pg/g (jaringan otak) di Solo (Jawa Tengah). Konsentrasi residu TCDD/TCDFs dalam daging sapi berada diatas batas maksimum residu (BMR) yakni sebesar 2 pg/g. Pakan ternak diketahui sebagai sumber utama kontaminasi dioksin pada daging. Perubahan makroskopik meliputi anemia, kaheksia, fibrosis hati, atropi jantung, penyumbatan rumen, konstipasi usus, perdarahan ginjal dan petechiae pada otak. Secara mikroskopis terlihat deplesia limpa, vakuolisasi interseptum, haemoragi dan akumulasi hemosiderin. Jantung mengalami degenerasi, fragmentasi dan pucat pada serabut otot dan pembengkakan inti sel. Hati terlihat pucat, degenerasi sel epitel dan kongesti. Paru – paru mengalami pneumonia, oedema pulmonum dan haemoragi ringan. Usus halus terlihat haemoragi dan infiltrasi sel mononuklear, neutrofil dan eosinofil. Otak mengalami perdarahan, perivascular cuffing dan terdapat intranuclear inclusion bodies. Hewan mengalami enteritis haemoragika, encephalitis dan degenerasi hati.

Kata Kunci: TCDDs, TCDFs, POPs, Produksi Ternak, Matriks, GC MS/MS

ABSTRACT

Sani Y, Indraningsih. 2015. Pathological changes of suspected tetrachloro dibenzo-ρ-dioxins/tetrachloro dibenzofurans toxication in beef cattle. JITV 20(3): 214-223. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1189

The contamination of tetrachlorodibenzo-ρ-dioxins (TCDDs) and tetra chlorinated dibenzofurans (TCDFs) may affect human or animal health such as cancer, reproductive failure, dermaltoxicities and neurologic effects. The present study describes the effects of TCDD/TCDFs contamination in feed to various tissues of beef cattle to which TCDD/TCDFs were detected byGC MS/MS. The results revealed that POPs (DDT, heptachlor, aldrin, dieldrin and endrin) as a precursor for dioxins were detected in all samples except drinking water. The total concentration of OC in soils was Nd – 42.73 μg/kg, grasses (3.30 – 27.66 μg/kg), well water (0.82 – 1.00 μg/kg), feed mill (3.90 μg/kg), sera (Nd – 13.08 μg/kg) and meats (Nd – 100.72 μg/kg). Futhermore, the TEQ residues of TCDDs/TCDFs in beef were 4496.66 - 20642.40 pg/g from Yogyakarta, and 717.13pg/g (beef) and 0.037 pg/g (brain tissues) from Solo (Central Java). The concentration of TCDD/TCDFs residues in beef was above the maximum residue limit (MRL) at 2 pg/g. Animal feeds is regarded as the main source of dioxins contamination in meats. Macroscopic changes were general anaemia, cachexia, fibrotic liver, athropic heart, ruminal impaction, constipated intestinal, haemorrhagic kidney, and ptechiae in the brain. Microscopically were depleted spleen vacuolation of interseptum, haemorrhages and accumulation of hemosiderin. Heart shows degeneration, fragmentation and pale cardiac muscle and swollen nuclei. Liver was pale, degeneration of epithelial cells and congestion. Lungs were pneumonia, oedema pulmonum and mild haemorrhage. Intestines showed haemorrhage and infiltration of mononuclear cells, neutrophyls and eosinophyls. Brain was haemorrhage, perivascular cuffs and intranuclear inclusion bodies. The animal was suffering from haemorrhagic enteritis, encephalitis, and hepatic degeneration.

Key Words: TCDDs, TCDFs, POPs, Animal Products, Matrices, GC MS/MS

INTRODUCTION

Tetrachloro dioxins and related compounds such as TCDDs/TCDFs and PCBs are toxic compounds of aromatic tetracyclic hydrocarbon derived from wastes of chemical processes or natural activities such as forrest fires, combustions, incinerators, household heating, and pulp and paper industry (McKay 2002; EC 2006a; EC 2006b). The contamination of dioxins becomes a public concern due to their undegradable properties in the environment and toxic effects in human health. As these compounds are highly non-polar lipophilic with longer biological half-lives, the dioxins may accumulate in food chains (Froescheis et al. 2000).The contaminated-food intake appears as the main source for human and animal exposures (Startin & Rose 2003). Chronic exposures in humans cause reproductive and developmental disorders, neurological effects, dermal toxicity, immunological changes and carcinogenic effects (Bencko 2003; Schwarz & Appel 2005; Wang et al. 2009; WHO 2010).

The exposures of dioxins in human as well as livestock are mostly resulted from intake of contaminated food (SCAN 2000; Bocioa et al. 2007), where they are regarded as the major source of exposure. Several studies have reported that the dioxins-contaminated feed were mainly due to (1) the use of contaminated clay; (2) the drying process of contaminated grass; and (3) the contaminated feed from treated timber using preservatives; and (4) the contaminated feed with wastes originating from industrial sources (Malisch 2000; Hoogenboom et al. 2009; Tlustos 2009a; Tlustos 2009b).

Animal products such as milk, eggs and meats are also significant sources for the contamination of dioxins (van Larebeke et al. 2001; Schmid et al. 2002). As milk is a nutritious source and the mammary gland may secrete several xenobiotic substances, it is known as risk factor for dioxins toxicities (Licata et al. 2004). The concentration of dioxins in milk depends on their concentration in pasture or commercial feed being consumed by cattle (Lorber & Winters 2007; Kleter et al. 2009).

The development of analytical techniques has facilitated the determination of dioxins in different matrices such as liquid samples of blood, milk and drinking water and solid samples of feed, tissues and soils. The techniques are therefore possible to monitor human exposure to dioxins and food safety of animal origin (Link et al. 2005). Monitoring of dioxins in foods of animal origin is important, since human exposures particulalry for younger age may cause adverse effect in brain development and the immune system (Weisglas-Kuperus et al. 2000). The present study is to asses the effects of TCDD/Fs contamination in feed to various tissues of beef cattle raised in a suspected dioxins contaminated areas in particular around an organic waste landfill area and a volcanic area, and to identify the potential sources of dioxins contamination in beef cattle.

MATERIALS AND METHODS

Sample collection

The present study was carried out to determine the concentration of dioxins in animal products and environmental matrices around the farms and to investigate their pathological changes in animal tissues. The locations of study was divided into two types following the previous study (Indraningsih & Sani 2014) including: (1) a volcanic area that had been errupted recently in Yogyakarta. The beef cattle farms were established surrounding the mountain and (2) an organic waste landfill is the place where beef cattle are being raised in this location in Central Java.The environmental matrices and blood samples were collected directly from the farms based on a tracing back of the previous study reported by Indraningsih & Sani (2014). Sample collection was carriedout in suspected dioxins and/or POPs-contaminated areas of Solo (Central Java) and Yogyakarta. Samples consisted of soils, water, public-waste ashes, pastures, blood of beef cattle and meat. The organic wastes were collected from the waste landfill in Solo consisting vegetables wastes, market wastes etc.

A number of 113 samples in this study was collected from Provinces of Yogyakarta (72 samples) and Central Java (41 samples), consisting 40 sera, 21 sera, 24 meats, 9 ovals, 6 soils, 6 water, 3 organic waste, 3 grasses and 1 feedmill of rice bran. Approximately 25-50 gram of matrices were collected from both areas and was kept in clean sampling bags or containers. About 200 – 500 ml of water samples were collected from irrigated water, wells and public water and kept in a sampling bottle individually. Blood were collected individually from beef cattle at the volume of 10 ml using plain–blood sample tubes. All field samples were processed for measurement of organochlorine pesticides or POPs using GC-ECD (Thermo Finigan). The dioxins were analysed from meats, animal tissues and feed using GC MS/MS (Thermo Scientific).

Solvents and standards

All solvents used were suprasolve grade and purchased commercially from Merck-Millipore (Germany) including hexane, toluene, dichloromethane and ethyl acetate. All standard solutions were the isomers of isotope $^{13}C_{12}$ (EDF-5999 of 99% purity) for internal standard solutions; a clean-up standard (EDF-6999 isotope $37Cl_4$) of 96% purity; a labelled

compound of EDF-8999 (Isotope stock solution); a calibration standard (EDF-9999-A isotope solutions CS_1 – CS_5) of 1/10 concentration; *n*-Nonane of 99% purity; and a precission and recovery standard solution (EDF-7999-10X) were purchased from Cambridge Isotope Laboratories (USA). The coloumn chromatography consisting alumina, carbon and silica were purchased from Thermo Scientific (Germany).

Sample-preparation procedure

The procedure of sample preparation is according to the method released by the EPA Method 1613 using FMS/Fluid Management System (EPA 1994). The method is for determination of tetra– through octa–chlorinated dibenzo-*p*-dioxins (TCDDs) and dibenzofurans (TCDFs) in water, soil, sediment, sludge, tissue, and other sample matrices by gas chromatography mass spectrometry (GC MS/MS). Samples were homogenised using dissection and/or mortar followed by an addition of hydromatrix powder, Na_2SO_4 anhydrate to dehydrate the samples and 20 µl labeled compounds following a method of EPA 1613. The FMS was then performed on dried products powder using an internal standard solution with *n*-Nonane as a solvent. This was followed by subsequent clean-up steps using a clean-up standard solution of 96% purity (EDF-6999 Isotope $37Cl_4$).

Instrumental analysis

The quantification of POPs or organochlorine pesticides residue was performed by GC-ECD (Thermo Finnigan) according to the methods described by Schenck & Wagner (1995) and Lehotay et al. (2005). The TCDD/Fs were quantified by GC MS/MS (Thermo Scientific) in MID mode and a Trace GC coupled to a MAT-95 XP mass spectrometer (Thermo Scientific). The GC MS/MS was supported with a CTC A 200S autosampler at 10000 resolving power (10% valley definition). Instrumental conditions and purity control criteria were according to the EPA 1613 method (EPA 1994). The limit of detection (LOD) for each congener was determined as the concentration in the extract which produced in two different ions and was monitored with a signal to noise ratio of 3:1 (CD 2004). The WHO-98 toxicity equivalent factors (TEF) were used to calculate the TEQ (Van den Berg et al.1994). Residues of TCDD/F were calculated using XCalibur program installed in the GC MS/MS.

Pathological examination

Clinical and pathological examination was also performed for beef cattle suspected to expose with POPs and/or dioxins and dioxin-like compounds. An exposed cattle was selected from a waste land fill area where the animal was grown on this area. Clinical examination was performed to the selected animal. Necropsy was carried out to examine pathological changes following a routine procedure for necropsy. Microscopic examination was carriedout on tissues showing lesions including liver, kidney, lungs, intestinals and brain. The tissues were stained with hematoxylin eosin (HE) and examined under light microscope.

RESULTS AND DISCUSSION

Analysis of persistent organic pollutants contamination in beef cattle

Organochlorines were detected in organic wastes, water, whole blood cells and tissues of beef cattle in Central Java. The POPs including DDT, heptachlor, aldrin, dieldrin and endrin were detected in organic wastes; faeces; meats, liver, spleen, heart, kidneys, lung, rumen and brains, but not in the intestines, lymphonodes and water (Table 1). There were four compounds of POPs detected in organic waste, such as DDT (Nd – 22.3 µg/kg); heptachlor (Nd – 1.9 µg/kg); Aldrin (1.5-7.3 µg/kg); and Endrin (Nd – 6.7 µg/kg). The POPs were also detected in faecal sample although the concentration was less than in organic waste, including DDT (1.2 µg/kg); heptachlor (0.7 µg/kg); aldrin (2.8 µg/kg); dieldrin (0.4 µg/kg); and endrin (2.1 µg/kg). The lesser amounts of POPs in faecal samples appear to be due to the metabolic process for contaminated feed intake. The compounds seem to be deposited in various tissues of the animal, and lesser amounts were excreted through urine and/or faeces as shown in Table 1. The dieldrin was not detected in organic waste but was found in faecal samples.

The residues of organochlorines were also found in meats, spleen and heart at the concentration range of 1.1-2.8 µg/kg (heptachlor); 0.8-7.3 µg/kg (aldrin); 0.2-0.6 µg/kg (dieldrin); and 0.2-3.1 µg/kg (endrin). These POPs were also detected in spleen and heart. POPs were not detected in intestines, but in ruminal content consisting DDT (2.2 µg/kg); heptachlor (5.4 µg/kg); dieldrin (0.4 µg/kg); and endrin (0.4 µg/kg).

Table 1. The concentration of organochlorines residue in different matrices collected from organic waste landfill area in Central Java

Samples (*n*)	Concentration of organochlorines (μg/kg)									Total OC (μg/kg)
	Concentration of POPs					Concentration of other OCs				
	DDT	Hepta.	Aldrin	Dieldrin	Endrin	Endosulfan	Lind.	Meth.	Chlor.	
OW (3)	Nd-22.3	Nd-1.9	1.5-7.3	Nd	Nd-6.7	0.4-39.1	1.0-2.5	Nd	Nd	3.9-77.9
DW (2)	Nd	Nd	Nd	Nd	Nd	Nd-9.6	Nd	Nd	Nd	Nd-9.6
Well water (1)	Nd	Nd	Nd	Nd	Nd	Nd	2.9	Nd	0.2	3.2
Faeces (1)	1.2	0.71	2.8	0.4	2.1	9.0	1.4	0.58	Nd	18.1
Meats (4)	Nd	1.1-2.8	0.8-7.3	Nd-0.4	Nd-3.1	Nd-3.7	0.3-0.8	Tt - 06	Nd	5.2-18.1
Livers (1)	Nd	1.5	Nd	Nd	Nd	Nd	1.9	Nd	Nd	3.4
Spleen (1)	Nd	1.1	2.2	0.2	0.2	Nd	Nd	Nd	Nd	3.7
Heart (1)	Nd	1.2	1.8	0.6	0.2	Nd	Nd	Nd	Nd	4.2
Kidney (1)	0.9	Nd	Nd	0.2	0.3	Nd	Nd	Nd	Nd	1.4
Lungs (1)	Nd	1.0	Nd	0.1	0.6	Nd	Nd	Nd	Nd	1.7
Lymphnodes (1)	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Intestines (1)	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Rumen (1)	2.2	5.4	Nd	0.4	0.4	Nd	Nd	Nd	Nd	8.5
Brain (1)	Nd	0.7	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.7

OW= Organic waste; DW= Drainage water; POPs= Persistent organochlorine pollutants; Hepta.= Heptachlor; (n)= Number of samples; Nd= Not detected; Meth.= Methoxychlor; ppb= Part per billion; Chlor.= Chlorpyrifos; Lind.= Lindane

Only heptachlor was detected in the liver as much as 1.5 μg.kg^{-1}.This may be due to the impaction of ruminal pylorus where undigested waste present in the stomach as shown in the patological examination. The presence of POPs residue such heptachlor (0.7 μg/kg) in brain tissues of the cattle should be taken into consideration. The dichloro diphenyl trichloroethane (DDT) was detected in organic wastes (Nd – 22.3 μg/kg), faeces (1.15 μg/kg), kidneys (0.9 μg/kg) and ruminal content (2.2 μg/kg). The organic wastes seem to be as the source of POPs contamination in beef cattle raised on this area.

Samples of Yogyakarta including soils, fodder and feedmill were collected from area around Mount Merapi that had been recently erupted between 2010 and 2011. Meats and blood samples were collected from a slaughtering house at Giwangan. Table 2 shows the concentration of organochlorine residues in environmental matrices and animal products collected in an erupted volcanic mount in Yogyakarta.

Although most POPs were detected in soils, grasses, feedmills, drinking water, sera and even meats, clinical cases of organochlorines toxicities were not found during the field visits. This was due to the concentration of POPs residues was below the maximum residue limits (≤200 μg/kg) which may not exert clinical signs of POPs toxicities in beef cattle.The animals that were suspected being toxicated by POPs were only showed cachectic, less activity, reduce in appetite and reduce in body weight.

The POPs (DDT, heptachlor, aldrin, dieldrin and endrin) were detected in all samples except drainage water. The total OC detected in soils was Nd – 42.7 μg/kg, grasses (3.3-27.7μg/kg), well water (0.8-1.0 μg/kg), feedmill (3.9 μg/kg), sera (Nd – 13.1μg/kg) and meats (Nd – 100.7 μg/kg). The dichloro diphenyl trichloroethane (DDT) was detected only in meats at the concentration of Nd – 19.8 μg/kg. While heptachlor was detected in soils, grasses, well water, sera and meats at the range of concentration residue was 0.1-35.8 μg/kg. Aldrin was between 2.6-6.6 μg/kg, dieldrin between 0.3-14.9 μg/kg and endrin between 0.8-2.7 μg/kg. This study indicates that the animal products of beef cattle in Yogyakarta were contaminated by POPs as detected in sera at the concentration of POPs

Table 2. The concentration of organochlorines residue in different matrices collected around an errupted volcanic mount in Yogyakarta

Types of OC	The concentration of organochlorine pesticide residues (μg/kg)						
	Soils (6)	Grasses (2)	Drainage water (1)	Well water (2)	Feedmill	Sera (20)	Meats (20)
POPs			Nd				
DDT	Nd	Nd	Nd	Nd	Nd	Nd	Nd-19.8
Heptachlor	Nd-35.8	0.8-3.3	Nd	0.1-0.7	Nd	Nd-3.8	Nd-20.5
Aldrin	Nd-2.6	Nd	Nd	Nd	Nd	Nd-6.6	Nd
Dieldrin	Nd	Nd	Nd	Nd-0.3	Nd	Nd-8.0	Nd-14.9
Endrin	Nd-2.7	Nd	Nd	Nd	2.0	Nd-0.8	Nd-2.4
Other OC			Nd				
Endosulfan	Nd-1.7	Nd	Nd	Nd	Nd	Nd-5.7	Nd-45.1
Lindane	Nd	Nd-0.9	Nd	Nd-0.7	1.9	Nd-0.3	Nd-15.4
Methoxychlor	Nd-0.3	Nd	Nd	Nd	Nd	Nd-5.0	Nd
Chlorpyrifos	Nd	1.6-24.4	Nd	Nd	Nd	Nd	Nd
Total OC	Nd-42.7	3.3-27.7	Nd	0.8-1.0	3.9	Nd-13.1	Nd-100.7

POPs= Persistent organochlorine pollutants; Ppb= Part per billion; Nd= Not detected; (n)= Number of samples

between 0.3-8.0 μg/kg and meats between 2.4-20.5 μg/kg. Soils appear to be the source of contamination of POPs in animal products with the concentration of POPs between 2.6-35.8 μg/kg.

Analysis of TCDDs/TCDFs in beef cattle farms using GC MS/MS

The analysis of TCDDs/TCDFs was carried out for animal tissues in particular meats collected from Central Java and Yogyakarta. Samples of animal tissues were collected from the necropsied cattle for a pathological study as above included kidneys, brain, meats, ruminal content and faeces. Meats were collected from Giwangan Animal Slaughtering House in Yogyakarta, where the origin of beef cattle was selected only from the areas suffered from the eruption of Mount Merapi recently. The determination of TCDDs/TCDFs was undertaken by GC MS/MS supported by automated sample preparation using 17 congeners as shown in Table 3 below. The results show that dioxins can be detected in all samples from both locations with a total TEQ, including Central Java: kidneys (99649.85 ρg/g), meat (1024.47ρg/g), ruminal contents (2016.51 ρg/g) and faeces (281313.86 ρg/g) and Yogyakarta: meats with a range between 6423.73 to 29489.14 ρg/g. The concentration of dioxins and dioxin – like in these samples seem to be above the maximum residue limit (MRL) stated by EC (2006) such as meat (4.5 ρg/g) and oval (12.0 ρg/g).

There were 4 of 17 congeners detected from meat of Central Java including 12378 – PeCDF, 23478 – PeCDF, 123478 – HxCDF, and 1234789 – HpCDF with a range of TEQ between 6.81 and 134.69 pg/g. Both 2378 – TCDF and 2378 – TCDD were not detected in this sample. Furthermore 7 congeners were detected in meats of Kulonprogo and Bantul (Yogyakarta) consisting 2378 – TCDD, 12378 – PeCDF, 23478 – PeCDF, 123478 – HxCDF, 123678 – HxCDF, 234678 – HxCDF and OCDF; 5 congeners in Ambarketawang (A): 2378 – TCDD, 23478 – PeCDF, 123478 – HxCDF, 1234678 – HpCDF, and OCDF; 4 congeners in Ambarketawang (B): 23478 – PeCDF, 123678 – HxCDD, 1234678 – HpCDF, and OCDF. The 2378 – TCDD was not detected in meats collected from 4 locations.

Food safety is becoming a vital issue at present day due to the incidence of dioxins contamination in food of animal origin and animal feed (Lorber & Winters 2007; Kleter et al. 2009). The concentration of dioxins and dioxin-like in meats and milk is depending on their concentration in pasture or other feed consumed by the animals. Animal products such as milk, eggs and meats are significant sources of dioxins and PCBs contamination for human and animals (van Larebeke et al. 2001; Schmid et al. 2002). The property of PCDDs/Fs is water soluble, but its solubility is increasing in organic solvents and fats with increasing chlorine content (Geyer et al. 2002; McKay 2002). Since their lipophilic nature and long biological half-

Tabel 3. The concentration of dioxins and dioxin-like in animal tissues from Central Java and Yogyakarta detected by GC MS/MS

Congeners	Concentration of dioxins in animal tissues (ρg/g)								
	Central Java					Yogyakarta			
	Solo					Kulonprogo	Bantul	Amb (A)	Amb (B)
	Kidney	Meat	Rumen content	Brain	Faeces	Meat (5)	Meat (3)	Meat (7)	Meat (5)
2378 – TCDF	6.08	0	0.96	0	21.19	0	0	0	0
2378 – TCDD	37.90	0	0	0	0	80.38	10.13	77.57	0
12378 – PeCDF	17.42	35.17	0	0.04	0	15.24	0.01	0	0
23478 – PeCDF	864.35	6.81	0	0	0	759.75	166.91	31.46	52.07
12378 – PeCDD	27.19	0	0	0	0	0	0	0	0
123478 – HxCDF	402.16	28.23	52.06	0	0	307.58	214.41	450.46	0
123678 – HxCDF	0	0	0	0	0	38.50	230.75	0	0
123789 – HxCDD	0	0	0	0	0	0	0	0	0
234678 – HxCDF	18471.65	0	0	0	0	8330	2621.70	0	0
123478 – HxCDD	0.61	0	0	0	0	0	0	0	0
123678 – HxCDD	0.61	0	0	0	0	0	0	0	4727.96
123789 – HxCDF	0	0	0	0	0	0	0	0	0
1234678 – HpCDF	102.0	0	350.28	0	0	0	0	4585.69	1117.78
1234678 – HpCDD	0	0	0	0	56241.58	0	0	0	0
1234789 – HpCDF	0	134.69	0	0	0	0	0	0	0
OCDD	0	0	0	0	0	0	0	0	0
OCDF	0.01	0	0.01	0	0	0.01	0.02	0.01	0.02
TOTAL TEQ ($pg.\mu l^{-1}$)	19929.97	204.89	403.30	0.04	56262.77	1284.75	3242.93	5145.20	5897.83
TEQ ($pg.g^{-1}$)	69754.50	717.13	1411.55	0.13	281313.86	4496.66	11350.26	18008.21	20642.40

Amb. = Ambarketawang
(n) = Number of samples

lives, the PCDDs/Fs and dioxin-like PCBs will acumulate in the food chain (Froescheis et al. 2000; Startin & Rose 2003). Most dioxins exposure in animals and humans are mainly through food intake (Liem et al. 2000). The chronic dioxins exposure in animals and humans causes a wide variety of toxic actions including reproductive and developmental effects, neurological and behavioral effects, dermal toxicity, immunomodulatory and carcinogenic effects (Bencko 2003).

The present study shows that dioxins and dioxin-like residues were detected in meats of beef cattle collected in Central Java and Yogyakarta, with a total TEQ between 1,284.75 and 5,897.83 pg/g and 204.89 pg/g respectively. The concentration of dioxins residues in these meat samples seem to be exceeded the maximum residue limit (MRL) stated by EC (2006a) as much as 4.5 pg/g. High concentration of dioxins residues in meat should be taken into consideration by the government and public since its toxicity effects in animal and human health. Animal feeds are presumed to be the source of dioxins contamination in meats as indicated from this experimental results that the samples were taken from an erupted of volcanic mount area in Yogyakarta and an organic waste landfill location in Central Java. This study also indicates that there was a correlation between POPs contamination detected in environmental matrices consisting soils, grasses etc and dioxins residue formation in meats.

Although the analytical equipment available in this institute is GC MS/MS triple quadropole (Thermo Scientific) that is usually used for screening analysis, the present results of dioxins residues in animal products and environmental matrices is the first report in Indonesia. The development of analytical techniques has facilitated the analysis of dioxins and dioxin-like in different samples including environmental matrices, blood, animal products and animal feeds. The techniques are therefore possible to conduct human exposure to dioxins and food safety monitoring by measuring dioxins and dioxin-like in blood plasma or serum (Covaci et al. 2002; Koppen et al. 2002; Link et al. 2005). This present study was conducted to asses potential sources of dioxins contamination in beef cattle raise around suspected contaminated areas such as natural disaster (erruption of volcanic mountains and floods) and public waste landfills.

Clinical and pathological effects of suspected dioxins contamination in beef cattle

Clinical and pathological examination was carriedout to a suspected dioxin-exposed beef cattle. The beef cattle was selected from a herd of cattle raised on an organic waste landfill in Central Java. Necropsy was undertaken to a selected beef cattle showing poor condition for pathological examination. The beef cattle was exsanguinated at both blood vessels: jugular vein and carotid artery. Clinical changes included general anemia, cachectic, anoretic, enlargement of abdominal flank as tympany-like, fatigue, diarhoea with smelt and dark faeces and dermatitis. Macroscopically showed the animal was pale and anemia, cachexia, shiny and watery skeletal muscle. Liver was mottled on the capsular surface, hardened and fibrotic (Figure 1). Heart was pale and athropy. Kidneys were ptechiae and hemorrhages. Gastrointestinal revealed as ruminal impaction due to undigested materials such as plastics and other harder materials, constipation and ballooning intestines containing watery materials and gas. Brain was congested and hemorrhages (Figure 2). Microscopic changes were seen in some organs of beef cattle such as liver, gastrointestinal tracts, kidney, spleen, lungs and brain. Liver was seen as degeneration of hepatic cells, congestion, accumulation of mononuclear cells around the portal tracts, and fibrosis (Figure 3). Reticulum showed necrosis of villi mucosa and tunika muscularis (Figure 4). Intestines were haemorrhagesand infiltration of lymphocytes, macrophages, neutrophyls and eosinophyls. Spleen was seen as deplesia of red pulp followed by vacuolisation of interseptum, haemorrhages and accumulation of hemosiderin. Heart was showing degeneration and fragmentation of cardiac muscle, swollen nuclei and pale cardiac muscle. Lungs were pneumonia, oedema pulmonum and mild haemorrhage. Brain was haemorrhage, congestion, perivascular cuffs and intranuclear inclusion bodies (Figures 5 and 6). The animal was then diagnosed as haemorrhagic enteritis, encephalitis, pneumonia, hepatic degeneration, cardiomyopathy and splenonecrosis.

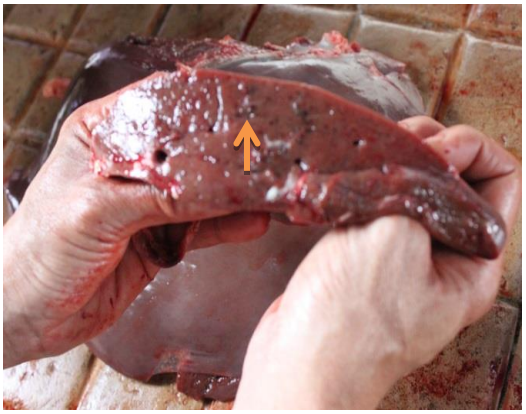

Figure 1. Liver of a beef cattle raised on a waste landfill showing ptechiae on the capsular surface, haemorrhagic and fragile (arrow)

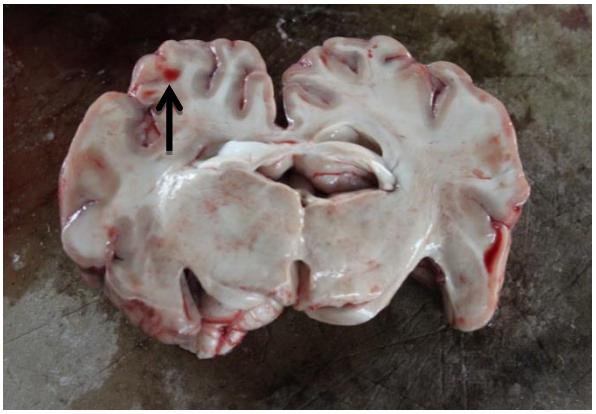

Figure 2. Brain of a beef cattle raised on a waste landfill showing petechiae on medula oblangata (arrow)

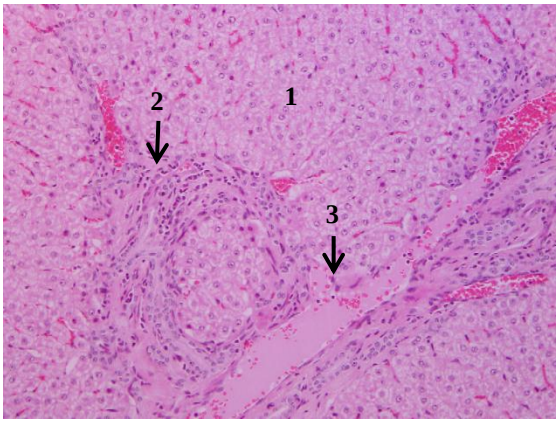

Figure 3. Liver of a beef cattle shows hepatic degeneration (1), infiltration of mononuclear cells (2), congestion and necrosis of blood vessels. HE.x200

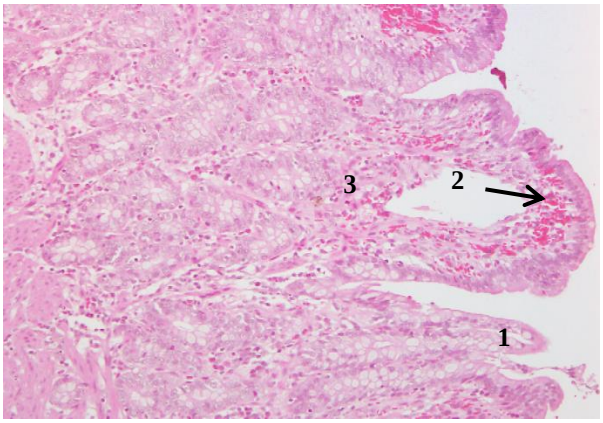

Figure 4. Reticulum of a beef cattle raised shows necrosis of villi mucosa (1) and hemorrhages (2), and accumulation of hemosiderin (3). HE x100

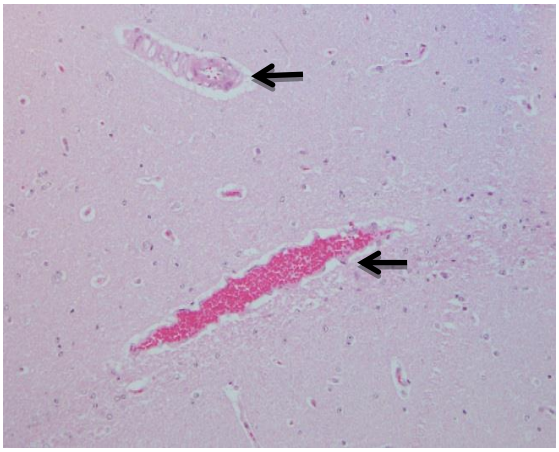

Figure 5. A brain tissue collected from beef cattle shows encephalitis indicated by perivascular cuffing and congestion (arrows). HE. 200X

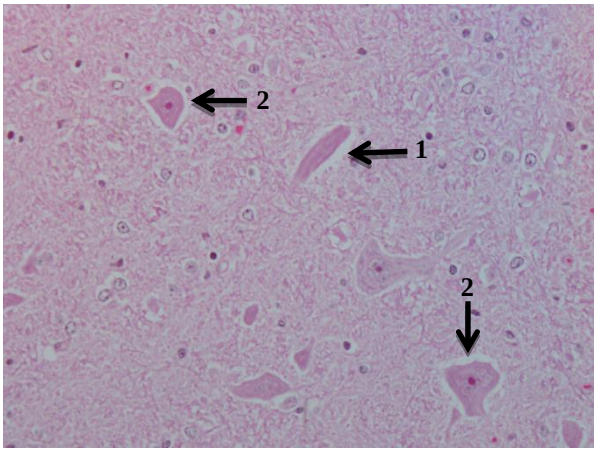

Figure 6. A brain tissue collected from beef cattle shows encephalomyopathy indicated by neuron necrosis (1), nucleolysis of neuron (1) and intranuclear inclusion bodies in neuron (2). HE. 200X

CONCLUSION

It is concluded that the TCDDs/Fs were detected in meats, ovals and environmental matrices, with a total TEQ between 6423.73 and pg/µg in Solo. The concentration of TCDDs/Fs residues in meats was exceeding the maximum residue limit (MRL) at 4.5 pg/g. High level of dioxins concentration in meat should be taken into consideration by the government and public since their toxicity effects in animal and human health. Animal feeds are regarded as the source of dioxins contamination in meats. Gross pathology of TCDD/Fs toxication was shown as general anaemia, cachexia, shiny and moist skeletal muscle, fibrotic liver, heart atrophy, renal haemorrhagy, and petechiae in brain. The results show that dioxins residues were detected in animal products and environmental matrices is the first report in Indonesia. Microscopically included deplesia of red pulp of spleen followed by vacuolisation of interseptum, haemorrhages and accumulation of hemosiderin. Heart shows degeneration and fragmentation of cardiac muscle, swollen nuclei and pale muscle. Liver was pale, degeneration of epithelial cells and congestion. Lungs were pneumonia, oedema pulmonum and mild haemorrhage. Intestines showed haemorrhage and infiltration of lymphocytes, macrophages, neutrophyls and eosinophyls. Brain was haemorrhage, perivascular cuffs and intranuclear inclusion bodies.

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Circulating H5N1 Virus among Native Chicken Living around Commercial Layer Farms

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ABSTRAK

Tarigan S, Indriani R, Ignjatovic J. 2015. Peredaran virus H5N1 pada ayam buras di sekitar peternakan ayam petelur komersial. JITV 20(3): 224-232. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1190

Sejak diterapkannya program vaksinasi kasus penyakit avian influenza (AI) H5N1 pada ayam pembibitan dan ayam ras petelur jarang terdengar. Penelitian in bertujuan menganalisis apakah redanya kasus tersebut berhubungan dengan hilangnya sumber infeksi di sekitar peternakan. Sampel serum dikumpulkan dari 421 ayam buras yang tinggal di sekitar peternakan ayam petelur di Kabupaten Cianjur dan Sukabumi, Jawa Barat pada Maret-April 2014. Antibodi virus AI H5N1 dianalisis dengan uji haemagglutination ihibition (HI), ELISA dan immunoblotting untuk mendeteksi antibodi terhadap haemagglutin virus H5N1, domain eksternal protein M2 (M2E) dan nukleoprotein (NP) virus AI. Sebanyak 8,6% dari ayam buras yang diperiksa seropositif terhadap virus AI berdasarkan satu atau lebih dari uji serologis. Hasil penelitian mengungkapkan bahwa virus H5N1 masih beredar pada ayam buras yang berkeliaran disekitar kandang ayam ras petelur. Sera yang positif dengan uji HI, M2E dan NP ELISA berturut turut 2,4%, 3,3% dan 3,8%. Tidak terlihat kesesuaian antara hasil satu uji dengan uji lainya. Penyebab ketidaksesuaian hasil tersebut diduga karena HI test, MM2e ELISA dan NP ELISA mengukur antibody yang berbeda yang kemunculan dan durasi masing masing antibodi tersebut berbeda. Kenyataan bahwa virus H5N1 masih beredar di sekitar peternakan ayam petelur menunjukkan bahwa ancaman virus AI masih membayangi peternakan ayam komersial dan karena itu vaksinasi dan biosekuriti yang ketat masih dibutuhkan.

Kata Kunci: H5N1, Ayam Buras, Petelur Komersial, Nucleoprotein, M2e, Uji HI

ABSTRACT

Tarigan S, Indriani R, Ignjatovic J. 2015. Circulating H5N1 virus among native chicken living around commercial layer farms. JITV 20(3): 224-232. DOI: http://dx.doi.org/10.14334/jitv.v20i3.1190

Soon after the application of vaccination programme against high pathogenic avian influenza H5N1 outbreak of the disease in breeder and commercial layer farms has diminished remarkably in West Java. This study aimed to investigate whether the H5N1 decline is related to the disappearance of source of infection around the farms. Serum samples were collected from 421 native chicken living around commercial layer farms in the Districs of Cianajur and Sukabumi, West Java in March-April 2014. Antibodies to avian influenza virus (AIV) H5N1 were measured using haemaglutination inhibition (HI), ELISAs and immunoblotting that measured presence of antibodies to the haemagglutin of H5N1 strain, as well as the M2e and nucleoprotein (NP) of all avian influenza viruses. Based on the combined results, 8.6% of the native chickens were seropositive to AI virus based on one or more of serological tests. This study provided serological evidence that H5N1 virus was still circulating among native chicken living around commercial layer farms. Many positive sera were however positive for antibodies in one test only: 2.4%, 3.3% and 3.8% by HI test, M2e and NP ELISA, respectively. It could be speculated that the incongruity of the results is due to the fact that HI, MM2e ELISA and NP ELISA all measure different type of antibodies and the duration of these antibodies in serum following infection with H5N1 differ. The fact that H5N1 virus is still circulating around commercial layer farms infers that the commercial farms are still under threat and therefore vaccination and strict biosecurity are still needed.

Key Words: H5N1, Native Chicken, Commercial Layer, Nucleoprotein, M2e, HI Test

INTRODUCTION

Indonesia is one of the countries hit hardest by the H5N1 virus. In no less than three years after its official declaration in 2004, the disease caused economic losses to the poultry industry of no less than 4.1 trillion rupiahs (Komnas Flu Burung, Antara 24 Maret 2008). Human deaths caused by the virus have been the highest in Indonesia totaling 165 deaths of 447 globally (www.who.int/influenza/ download July 2015).

Since its first appearance in Indonesia in 2003, HPAI H5N1 spread rapidly leaving little options for the government except to implement mass vaccination. It was decided that vaccination in sector 1, 2, and 3

commercial poultry was the responsibility of the farm's owners, whereas the sector 4 poultry became the responsibility of the government. To date vaccination of commercial poultry has been successfully applied with vaccination coverage of nearly 100%. As a result, H5N1 outbreaks in sectors 1, 2 and 3 have dropped remarkably (Siregar et al. 2007). In sector 4 poultry, on the other hand, mass vaccination was discontinued because of the difficulty in its implementation (Siregar et al. 2007). Due to the lack of control measures, it is suspected that H5N1 virus readily spread among poultry in sector 4, which have become latent treat to the nearby commercial poultry.

Vaccination against influenza virus can protect chickens from mortality and clinical disease but not always against infection (Suarez 2005). This means that if vaccinated commercial poultry are challenged by H5N1 originating from surrounding sector 4 poultry, subclinical infection is the most likely outcome. We have investigated this possibility by carrying out a longitudinal study in commercial layer farms in West Java and Jogjakarta provinces. Extensive year-long investigation showed that there was no indication of H5N1 infection on the studied farms. One possible cause for the absence of infection in those layer farms is the absence of virus challenge and by extension also, the absence of infection in native chickens living around farms. This study, which was carried out at the end of our longitudinal study, aims at investigating serologically evidence for the existence of AI virus infection in village, free range or native chickens living in villages around commercial layer farms. The study indicated that H5N1 infection was still occurring among many native chickens living around commercial layer farms albeit at low prevalence.

MATERIALS AND METHODS

Native chickens

Native chickens living within about 1 km radius from six commercial layer farms (3 farms in Sukabumi and 3 in Cianjur districs) were bled in March - April 2014. The sample collection was facilitated by the officers of District Animal Health Services who organized and asked the village farmers not to release their chicken at the days of sample collection. A simple questionnaire was prepared to ease recording on (1) the age group of each bird bled, (2) the name and address of the owner, (3) number of poultry they owned, (4) if disease or death in poultry had occurred in the neighborhood, (5) whether they vaccinated their chicken against avian influenza, (6) if any of their family or neighborhood worked on commercial layer farms and (7) whether they bought culled chickens from any layer farms.

Serological testing

Antibodies to AI virus in collected sera were used as an indication of infection by virus, and thus indirectly of the presence of AI virus the population. Initially, three serological tests were used. (1) A non-commercial validated competitive nucleoprotein of type A influenza viruses, irrespective of subtype. Testing was carried out according to the protocol provided by the test developer (AAHL, Australia) with a cut-off value of ≥60% inhibition as suggested. (2) The haemagglutinin inhibition (HI) test, performed according to a standard protocol (OIE 2014) was used to detect antibody to the haemagglutinin specific for the H5 subtype, with a cut-off value for positivity of 4 log2. The HA antigen for the HI test was prepared from a local isolate A/Ck/WJ/PWT-WIJ/2006 (H5N1). (3) A MAP-M2e ELISA was used to detect antibody to external domain of M2 protein (M2e) of AI (H5N1) virus. The protocols for this test has been described previously (Tarigan et al. 2015). Briefly, diluted sera were added to the 96-well microtitre plate that previously had been coated with with 4-symmetry-branched-M2e peptide. Antibody specifically bound to the M2e peptide was probed with HRP-anti-chicken conjugate. The cut-off value for a positive sample was 0.1

Serum samples that were positive with any of the three tests were further analysed with immunoblot or ELISA using relevant recombinant proteins of influenza virus. The recombinant proteins expressed in mammalian cells were obtained from Sino Biologicals Inc. China. The recombinant proteins included full size, polyhistidine-tagged nucleoprotein from Influenza A H1N1 strain (A/Puerto Rico/8/34/Mount Sinai) (cat. no. 11675-V08B) and polyhistidine-tagged extradomain of heamagglutinin from Influenza A H5N1 (A/Indonesia/5/2005) GenBank Accession no. ABW06108.1) (Met 1 – Gln 531) with cleavage site mutated (RESRRKKR to obtain noncleaved H1+H2) (cat. no. 11060-V08H1).

Indirect NP and H5 ELISAs

Each recombinant protein was diluted in 0.1 *M* carbonate buffer (pH 9.6) at 2 µg/ml then used to coat microtitre plates (Nunc maxisorp) overnight at 4°C. After blocking with non-fat-skimmed milk (5 mg/ml, 2 hrs), serum samples and controls, diluted in PBST (PBS pH 7.2, 0.05% Tween-20) at 1:100, or serially diluted when indicated, were added and incubated at 37°C for 1 hr. Serum controls included serum from influenza-free chicken (negative control) and serum from chicken that had been vaccinated and infected with a H5N1 virus (A/chicken/West Java/Sbg-29/2007 (GenBank accession no. KC831453.1)) (positive control). After washing 4 times with PBST, anti-chicken-IgG-HRP

conjugate (Sigma Co. Singapore) diluted at 1:4000 was added then incubated at 37°C for 1 hr. After washing 4 times, chromogenic (ABTS) substrate was added and the absorbance was recorded with a microtitre-plate reader. The OD of a sample was standardized with the following formula: (OD sample-OD negative control)/(OD positive control – OD negative control). The cut-off value was 0.1 for both indirect NP and H5 ELISAs.

Immunoblotting

Recombinant proteins diluted at 50µg/ml in sample buffer, were separated in the 10% -acrylamide-separating gels on SDS PAGE. Proteins from the gels were transferred onto a nitrocellulose membrane. After blocking with skimmed milk (5 mg/ml, 2 hrs), serum samples and controls diluted at 1:200 in PBST, were added and incubated at 25°C for 2 hr. After washing 4 times with PBST, anti-chicken-IgG-HRP conjugate (Sigma Co. Singapore) diluted at 1:4000 was added then incubated at 25°C for 2 hrs. After washing 4 times, chromogenic (DAB) substrate was added to probe bound antibody.

RESULTS AND DISCUSSION

The number of sera available for this study was collected from 421 native chickens (40% growing birds (2-6 months old) and 60% adult (>6 months) from 16 villages, around 6 layer farms, within a distance of ≈ 1 km from the farms (Table 1). They were typically backyard chickens that were free to roam in the neighborhood and around the layer farms during the day. Some owners of native chicken or their neighbors worked on the layer farms and occasionally brought home culled chickens from layer farms and raised them together with their native chickens. However, non of these birds were bled.

Some chickens could not be bled because they had been freed by the owner before the survey teams arrived, and chicks less than 2 months old were not bled. Although the exact number of native chickens around the layer farms were unknown, it was estimated that at least 25% of the total population was successfully bled.

Thirty six (8.6%) of the samples were positive in one or more of the three tests, AAHL-NP ELISA, M2e

Table 1. The number and location of native chickens bleed for the serological surveillance

District	Related farm	Village	No. chicken	
			Village	Farm
Cianjur	1. CCA*	1. Legok Karso	61	96
		2. Ciherang	35	
	2. n/a[#]	3. Ciremis	11	11
	3. CKR	4. Cinangka	57	84
		5. Bedahan	27	
	4. CHA	6. Karang Anyer	42	52
		7. Cipolong	10	
Sukabumi	5. STA	8. Tangkil Waru	18	32
		9. Tangkil Lande	14	
	6. SCR	10. Sasagara	21	97
		11. Cikaung	7	
		12. Cikaret hilir	10	
		13. Cibaringbing	59	
	7. SPU	14. Purwasari	17	45
		15. Sirnabakti	28	
	8. n/a	16. Tapos	4	4
Total			421	421

(*) anonymised name; (#) indicates that the sampling was not related to any layer farm. These two villages were sampled because a HPAI outbreak in ducks was reported to have occurred within them

Table 2. Results of the examination of sera from native chicken with AAHL NP ELISA, MM2e ELISA and HI test

Test	Positive	Negative
MM2e ELISA	14 (3.3%)	405 (96.7%
NP-ELISA	16 (3.8%)	403 (96.2%)
HI test	10 (2.4%)	409 (97.6%)
M2e and HI and NP-ELISA	0 (0.0%)	419 (100.0%)
M2e or HI or NP-ELISA	36 (8.6%)	383 (91.4%)
M2e and HI	0 (0.0%)	419 (100.0%)
M2e and NP-ELISA	1 (0.2%)	418 (99.8%)
HI and NP ELISA	3 (0.7%)	416 (99.3%)

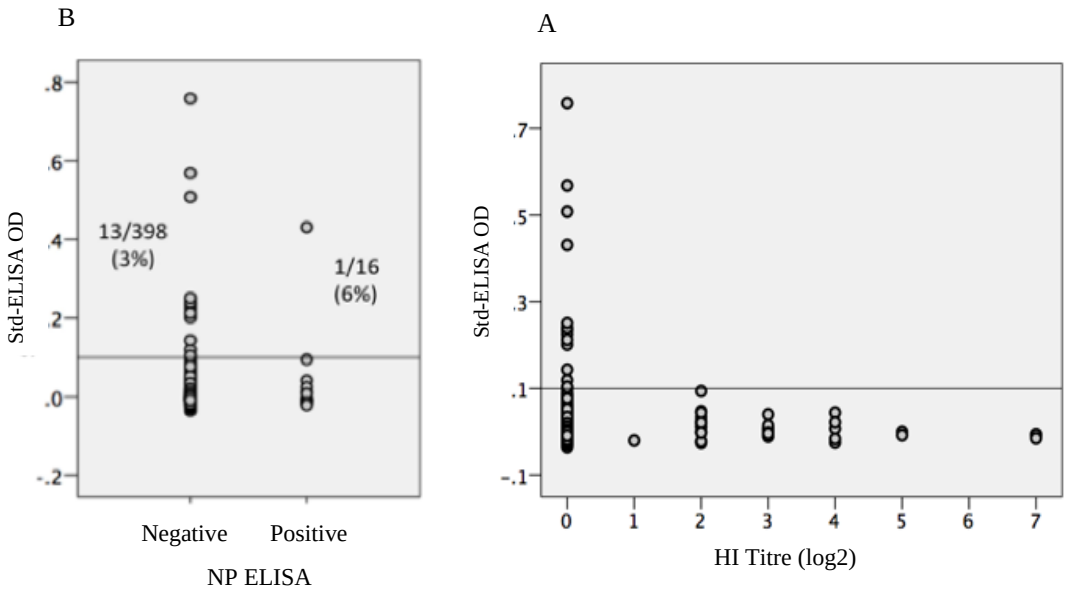

Figure 1. Agreement between the results of MM2e-ELISA with NP-ELISA (A) and MM2e ELISA with HI test (B) on 420 sera collected from native chicken that roamed nearby commercial layer farms

ELISA or HI test. However, no serum was positive in all three tests and, the number of sera that were positive with any two tests was very small (Table 2). Since the AAHL-NP ELISA detect antibody to NP protein, all sera from birds that had been infected by any influenza A viruses should have been positive in the test. All sera that were positive in HI test or M2e ELISA should also be positive in the NP ELISA. However, only 3/10 sera in this study that were positive in HI test were also positive in AAHL-NP ELISA.

The seropositivity with the AAHL-NP ELISA and HI test could not have been due to vaccination, because no vaccination against AI had been carried out in those villages for the last three years. Unlike the NP ELISA and HI test, M2e ELISA detect M2e antibody that is induced only by infection with influenza virus. There were 14 sera (3.3%) positive for M2e antibodies. Four sera had OD of >0.1 – 0.2, six had OD of >0.2 - 0.3 three had OD of >0.3 -0.5, and one serum had high (>0.7) OD. There was only one of the M2e-positive serum that was positive in NP ELISA, and none were positive for HI test (Table 1, Figure 1).

The M2e-positive sera were not clustered in any of the villages or around a particular layer farm (Figure 2). The percentage of M2e positive sera in mature chickens was twice as high as that in young chickens (results not shown).

Recombinant-NP-based assays

Examination of sera that were positive in either HI test, AAHL-NP and MM2e ELISAs revealed that all of those positive in AAHL-NP ELISA were also positive in the indirect NP ELISA. Two AAHL-NP-ELISA-positive sera with high OD (0.788 and 0.956) in indirect NP ELISA were negative in M2e ELISA (standardized

M2e-ELISA ODs= -0.016 and -0.015) (Table 3). Examination of the NP-ELISA-positive sera with immunoblotting indicated that the majority of sera (7/10) recognized the recombinant nucleoprotein. One sample (bird# 365), however, reacted unexpectedly because despite being positive in both AAHL- and direct-NP ELISAs the serum did not recognized the NP protein (Table 3, Figure 3A).

Recombinant-H5-based assays

Results of indirect-H5 ELISA on serially diluted sera are presented in Figure 4. All sera that were negative in HI test, M2e and AAHL-NP ELISAs were also negative in the indirect-H5 ELISA. One serum (from bird# 450) that had a HI titre of 7 log2 had a high OD even after the serum was diluted at 1:3200. The other serum with log2 Hi titre of 4 (from bird #365) had a much lower OD at any serum dilution. All sera that were negative in HI test were also negative in direct H5 ELISA.

The result of the indirect-H5 ELISA was in agreement with that of the immunoblot, as all sera with standardised OD ELISA's of ≥0.048 were positive whereas those of ≤-0.041 were negative in the immunoblot (Table 2, Figure 3B). However, the antibody titres as determined by HI test were poorly correlated with the ELISA's OD and the immunoblot signal. For example, sera # 261 and # 262 that both had HI titre of 7 log2, and sera # 231 with 5 log2 were all negative in the ELISA and immunoblot. Whereas, sera # 358 and #453 that both were negative in HI test were positive in both the ELISA and immunoblot (Table 2, Figure 3B).

Regardless of the inconsistency of the results given by different tests on many sera, there were at least two sera that the test results were consistent. The results of all tests on serum from bird# 450, except MM2e ELISA, were all strongly positive. The results of tests on serum from bird # 365 were comparable with those on serum from bird# 450, except that the serum failed to recognize the NP in immunoblot (Table 2, Figure 3B).

Table 2. Results of the examination of sera from native chicken with NP ELISA, MM2e ELISA and HI test

Bird	M2e ELISA's OD	AAHL-NP ELISA	Direct-rec-NP ELISA's OD	W'blot-rec NP	HI- titre (log2)	Direct-rec- H5 ELISA's OD	W'blot rec H5
358	0.568	-	-0.041	+	0	0.079	+
380	0.508	-	0.001	+	0	-0.091	-
453	0.251	-	0.027	+	0	0.048	+
343	0.094	-	-0.009	-	0	-0.041	-
231	0.000	-	-0.054	+	5	-0.050	-
261	-0.006	-	-0.038	+	7	-0.048	-
262	-0.007	+	-0.066	+	7	-0.068	-
297	-0.008	-	0.048	-	5	0.324	+
450	-0.015	+	0.956	+	7	0.991	+
365	-0.016	+	0.788	-	4	0.948	+

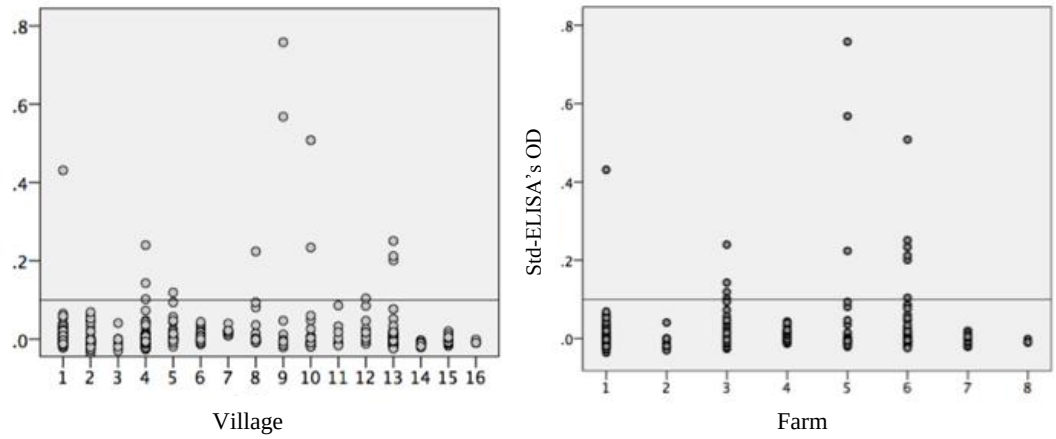

Figure 2. The NP-ELISA positive samples were spread randomly within the village (A) or around the layer farms (B). Note: the identities for the farms and villages are presented in Table 1

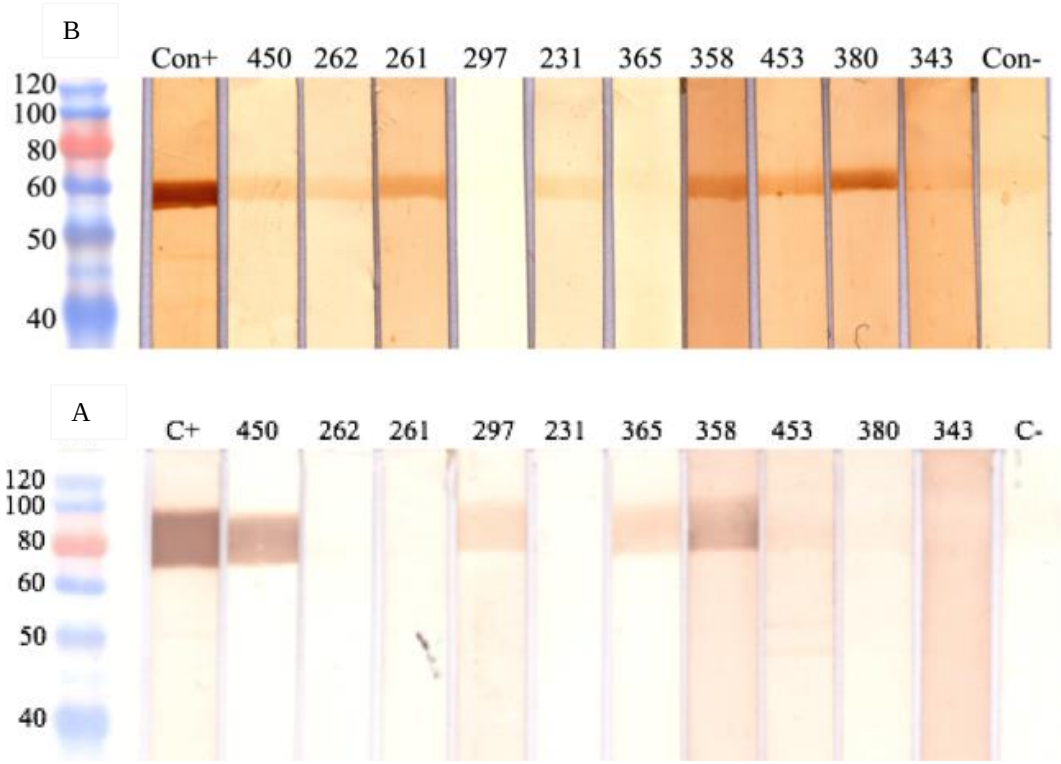

Figure 3. Recognition of nucleoprotein (A) and hemagglutinin H5 (B) by sera from native chicken that are seropositive in HI test, NP and M2e ELISAs

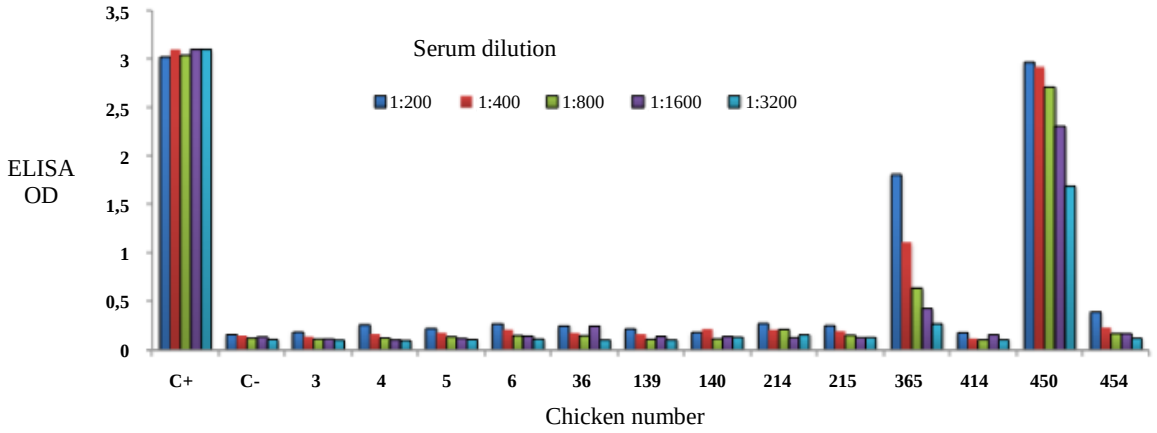

Figure 4. Direct-H5 ELISA on titrated sera from native chicken. Sera # 3, 4, 5, 6, 36, 139,140, 214 and 215 were negative on HI test, M2e ELISA and AAHL-NP ELISA, serum #414 was positive M2e (OD=0.201), serum# 454 was positive AAHL-NP ELISA, sera # 365 and 450 see table 2. C+ serum from chicken that had been 2 times vaccinated follow by infection with H5N1, C- serum from bird naïve to H5N1

Discussion

This study provides serological evidence of the circulating H5N1 virus among native chicken living around commercial layer farms. As shown in this study, 8.6% of the native chickens were seropositive to AI virus based on one or more serological tests. Despite the difficulty in interpretation of the test results on many positive sera because different tests did not support one another, seropositivity in at least two birds has most likely resulted from infection by subtype H5 influenza virus. This is because sera from birds contained antibodies to the NP as indicated by high OD in indirect ELISA, strong inhibition in competitive ELISA and reaction to the recombinant NP in immunoblot assay. Antibody to HA5 was evidenced by high HI titres and high OD in indirect H5 ELISA and strong reaction to the recombinant H5 in immunoblot assay. Since H5 subtypes, other than H5N1, have been unknown in Indonesia, and the H5N1 subtype has been endemic in this country since 2003, the seropositivity in those birds is likely to be caused by H5N1 virus subtype.

The fact that H5N1-seropositive chickens were found in native chickens living around commercial layer farm has at least two important implications. Firstly, layer farms may be under threat from H5N1 virus challenge originating from their immediate surroundings. The absence of the disease in commercial farms for the last several years may be attributed to the effectiveness of biosecurity measures applied and vaccination. Secondly, seroconversion to H5N1 virus in those birds, without being preceded by apparent mortality or clinical disease, in the population of the native chicken suggest that the pathogenic trait of the H5N1 virus may have waned considerably. Although low pathogenic H5N1 exists in nature, its existence in poultry is uncommon (Duan et al. 2007; Pei et al. 2009; Kim et al. 2011; Van Borm et al. 2011; Ping et al. 2012). In H5N2 subtype, mutation of LPAI into HPAI and existence of both LPAI and HPAI in the same poultry farms have been well characterized in poultry in USA (Swayne 2008). As far as we are aware similar incidences of H5N1 subtype have not been found. It is true that the HPAI H5N1 is believe to mutate from a LPAI but where and when the mutation took place and which H5N1 LPAI as the progenitor of the H5N1 HPAI are unknown (Wan 2012).

The notion that the circulating HPAI H5N1 strains have waned in pathogenicity supported by the reduction in the number of reported outbreak of AI in native chickens in Indonesia. The decline of the H5N1 cases was not caused by any control measures applied. There are no control measures that had been applied in native chickens attributed to declining of the H5N1 outbreak. At the peak of H5N1 outbreak, mass vaccination in sector-4 poultry had been attempted but discontinued as it was not feasible (Siregar et al. 2007). Application of biosecurity in backyard poultry to an extent effective to abate the H5N1 infection is difficult to achieve (Conan et al. 2012).

Until December 2008, Sukabumi and Cianjur were the districts with the highest cases of H5N1 in West Java, and West Java was the province with the highest number of H5N1 outbreaks in Indonesia (Yupiana et al. 2010). However, since 2009 outbreaks of AI in chicken in those districts have been rarely reported and occurrence of H5N1 outbreak in the whole country has dropped significantly (www.keswan.ditjennak.pertanian.go.id.). The increase in the number of H5N1 outbreaks since 2012 in Indonesia is related to the

spread of the new H5N1 clade, clade 2.3.2.1, (Dharmayanti et al. 2014). However, this new clade H5N1 caused disease and mortalities mostly in ducks, whereas poultry including, native chicken, apparently were not affected (Empres 2014).

The discrepancy of results given by different tests on the same sera were unexpected because all tests used in this study had been validated before use. The AAHL-nucleoprotein ELISA is a competitive ELISA that had been proved to be sensitive and specific for detection of antibody to the NP of type-A influenza viruses in birds and mammals (Sergeant et al. 2009; Sergeev et al. 2013) and was used in the surveillance of AI in wild and domesticated birds in Australia (OCVO 2010).

The MM2e ELISA was shown to be highly specific based on a validation study using chicken serum samples from vaccination and challenge trials (Tarigan et al. 2015). Since not all birds infected with the influenza virus seroconvert to M2e, the percentage of native chicken that had been infected by influenza virus must therefore been higher than the M2e seroprevalence of 3.3% (Lambrecht et al. 2007; Kim et al. 2010; Hemmatzadeh et al. 2013; Tarigan et al. 2015).

The result of HI test in this study were more incongruent in comparison to other tests. For example, two sera with high (7 log2) HI titres were negative in the indirect ELISA and immunoblot using recombinant H5 encoded by gene derived from an Indonesian isolate. The HI test in this study was performed in a nationally accredited diagnostic laboratory that routinely performs HI testing.

The cause of the incongruity of the results provided by the well-validated HI test, nucleoprotein and MM2e ELISA is unknown. The incongruent results caused difficulty in determining the seroprevalence of AI in the native chicken. A more sensitive and specific test is needed for native chicken. As far as we are aware, similar problem has not been reported. This is probably because most serological studies usually use only one test, either HI test or ELISA (Nasreen et al. 2013; Chang et al. 2014). A possible reasons is that HI, MM2e ELISA and NP ELISA all measure different type of antibodies and it is likely that the duration of these antibodies in serum following infection also differ. The HI antibodies last long in vaccinated chickens (Meulemans et al. 1987) whereas the M2e antibodies last only about 8 weeks (Tarigan et al. 2015), whereas the duration of NP antibodies is unknown.

In summary this study shows that H5N1 virus influenza is still circulating among native chicken near commercial layer farms. Infection in these chickens is subclinical probably because the pathogenicity of the virus is waning. Since the commercial layer farms are still under threat, vaccination and strict biosecurity are still necessary. The results of HI test, NP and M2e ELISAs are not in agreement suggesting that a more sensitive and specific test is needed for surveillance of AI in native chicken.

CONCLUSION

To sum up, this study shows that H5N1 virus influenza is still circulating among native chicken near commercial layer farms. Infection in these chickens is subclinical probably because the pathogenicity of the virus is waning. Since the commercial layer farms are still under threat, vaccination and strict biosecurity are still necessary. The results of HI test, NP and M2e ELISAs are not in agreement suggesting that a more sensitive and specific test is needed for surveillance of AI in native chicken.

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