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# Jurnal Ilmu Ternak dan Veteriner

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**BADAN PENELITIAN DAN PENGEMBANGAN PERTANIAN**
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## PREFACE

On February 10, 2016, Indonesian Journal of Animal and Veterinary Sciences (IJAVS) received a certificate from Index Copernicus International (ICI). IJAVS was indexed and reported in the ICI Journal Master List. ICI Journal Master List Reports contain information of more than 3400 scientific journals from around the world. Index Copernicus Value (ICV) for IJAVS is 62.49. We hope with this ICV authors will be more confident to published their research results in IJAVS.

In this edition, volume 20 no. 4 December 2015, we proudly present articles from various disciplines such as Animal reproduction; feed and nutrition technology; forages technology; and veterinary technology. The articles published in this edition are: "Microencapsulation of bovine spermatozoa: Cryopreservation of microencapsulation sperm using glycerol"; “Gas production and rumen fermentation characteristics of buffalo diets containing by-product from some sorghum varieties"; "Role of sweet orange (*Citrus sinensis*) waste in lowering the meat cholesterol and fat of Padjadjaran sheep"; "Voice characteristics of some sheep: Utilization to estimation of genetic distance"; “Carbohydrate and protein digestion on Palm Kernel Cake by Mannanase BS4 and papain cocktail enzymes"; "Trypanocidals effectivity against some isolates of *Trypanosoma evansi* propagated in mice"; “Endemicity of avian influenza in ducks living around commercial layer farms” and "Molecular identification technique of *Trypanosome evansi* by Multiplex Polymerase Chain Reaction"

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

Chief Editor

Bogor, December 2015

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# Microencapsulation of Bovine Spermatozoa: Cryopreservation of Microencapsulation Sperm using Glycerol

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## ABSTRAK

Kusumaningrum DA, Purwantara B, Yusuf TL, Situmorang P. 2015. Mikroenkapsulasi spermatozoa sapi: Kriopreservasi mikroenkapsulasi spermatozoa menggunakan gliserol. JITV 20(4): 233-241. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1240

Kriopreservasi spermatozoa merupakan proses preservasi spermatozoa yang dilakukan pada suhu yang sangat rendah. Krioprotektan intraselular gliserol merupakan krioprotektan yang paling sering dikombinasikan dengan pengencer Tris sitrat yang mengandung 20% kuning telur (TSKT), sementara TSKT juga digunakan sebagai media enkapsulasi bersama dengan 1,5% alginat. Pengaruh alginat dalam mikroenkapsulasi dan gliserol terhadap daya hidup spermatozoa (motilitas = %M, spermatozoa hidup = %H dan Tudung akrosom utuh = %TAU) diteliti dalam dua tahapan penelitian. Pada penelitian pertama, pengaruh alginat dan proses mikroenkapsulasi dipelajari dengan tiga perlakuan yaitu: 1) TSKT ditambah dengan semen; 2) TSKT-Alginat ditambah dengan semen dan 3) TSKT-Alginat ditambah semen dengan mikroenkapsulasi; yang diikuti dengan proses kriopreservasi spermatozoa secara konvensional menggunakan TSKT 7% gliserol. Pada penelitian kedua pengaruh konsentrasi gliserol (5 dan 7%) dan waktu ekuilibrasi diteliti menggunakan disain faktorial 2 x 2. Proses pembentukan mikroenkapsulasi berpengaruh nyata terhadap persentase motilitas spermatozoa (%M) dan tudung akrosom utuh (%TAU) pada post-thawing, yaitu 44,17 dan 50,83% untuk %M dan 79,33 dan 83,59% untuk %TAU berturut turut untuk mikroenkapsulasi dan kontrol. Dijumpai adanya fakta bahwa alginat berperan sebagai krioprotektan ekstraseluler karena kemampuannya dalam mencegah penurunan daya hidup spermatozoa selama proses kriopreservasi. Rata-rata persentase penurunan dari %M dan %TAU pada TSKT-Alginat 15,97 dan 6,44%, lebih rendah dibandingkan kontrol-TSKT yaitu 23,8 and 7,37%. Dijumpai adanya kenyataan bahwa alginat berperan sebagai krioprotektan ekstraselular karena kemampuannya melindungi spermatozoa selama proses pembekuan, sehingga persentase penurunan dari %M dan %TAU pada TSKT-alginat (15,97 dan 6,44%) lebih rendah dibandingkan dengan kontrol-TSKT yaitu 23,80 dan 7,37%. Tidak dijumpai adanya pengaruh yang nyata dari konsentrasi gliserol (5 dan 7%). Disimpulkan, proses mikroenkapsulasi menurunkan daya hidup spermatozoa, alginat berperan sebagai krioprotektan ekstraselular sehingga kriopreservasi dari mikroenkapulasi spermatozoa dapat dilakukan menggunakan gliserol 5 atau 7%.

**Kata Kunci**: Spermatozoa, Alginat, Mikroenkapsulasi, Gliserol, Waktu Ekuilibrasi

## ABSTRACT

Kusumaningrum DA, Purwantara B, Yusuf TL, Situmorang P. 2015. Microencapsulation of bovine sperm: Cryopreservation microencapsulation of sperm using glycerol. JITV 20(4): 233-241. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1240

Cryopreservation of spermatozoa has been used to preserve spermatozoa in very low temperatures. Glycerol is intracelullar cryoprotectant usually used in Tris citrate containing 20% egg yolk (TCEY), while TCEY-1.5% alginate was used as encapsulation media. The effect of alginate in microencapsulation process and glycerol concentration on viability spermatozoa (motility (%M), live sperm (%L) and intact apical ridge (%IAR) were studied in two steps. In the first step, the effect of alginate and microencapsulation process was studied. Three treatments of this step were: 1) TCEY add with semen (as control), 2) TCEY-Alginate add with semen, 3) TCEY-Alginate add with semen and microencapsulated, followed by conventional sperm cryopreservation using TCEY 7% glicerol. The second steps were done to evaluate the effect of two glycerol concentrations (5 and 7%) and two duration of equilibration time (3 and 4 hours). Result of these experiments showed, that microencapsulation was significantly alter the percentage of post-thawing motility (%M) and intact apical ridge (%IAR). The motility of microencapsulated sperm vs control was 44.7 and 50.83% respectively, whereas IAR 79.33% and 83.50% on microencapsulated sperm and control. There was evidence that alginate act as extracelullar cryoprotectant by protecting sperm during freezing. The mean of decreasing percentage of %M and %IAR in TCEY-Alginate were lower (15.97 and 6.44%) than control (23.80 and 7.37%). The effect of glycerol concentration and equilibration time on the viability of sperm was not significant different. There was no significant interaction of glicerol and equilibration time on the viability of spermatozoa. In conclution, microencapsulation processes altered the viability of sperm, alginate had an important roles as extracelullar cryoprotectant. Moreover the cryopreservation of microencapsulated sperm might be done using 5 or 7% glycerol in 3-4 hours duration of equilibration time.

**Key Words:** Sperm, Alginate, Microencapsulation, Glycerol, Equilibration Time

## INTRODUCTION

The cryopreservation of sperm is preservation process of sperm cell performed at very low temperature (commonly at -196°C by using $N_2$ liquid). Temperature decreasing process causes changing media environment (osmolatiry increase and ice crystal formation) which damaging the cell.

During cryopreservation process, sperm undergo stress due to temperature decrease. Temperature stress will be decrease the function and fertility of sperm. Some sperm cryopreservation problem were decreasing of viability of sperm more than 50%, induction of acrosome reaction, decrease of motility and problem of fertility (Chavero et al. 2006; Wongtawan et al. 2006). Sperm damage occurred through two mechanisms: due to mechanic process through ice crystal formation and due to ostmotic effect and solute concentration known as solute effect (Wowk 2007). Addition of cryoprotectant agent was conducted to decrease the lethal effect of the ice crystal formation and solute effect.

According to ability to penetrete cell membrane, cryoprotectant was divided as intracellular and extracellular cryoprotectant. Intracellular cryoprotectant can penetrate membrane cell, such glycerol, methanol, dimethyl sulfoxide (DMSO), 1.2 propanediol, butanediol, acetamide, propylene glycol. Extracellular cryprotectant have high molecular weight, can't penetrate into the cell and works outside the cell. Lactose, raffinose, mannase, polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), trehalose and proteins such as lipoprotein of egg yolk ware extracelullar cryoprotectant. (Wowk 2007; Lemma 2011).

Glycerol (C3H8O3), alcoholic sugar with BM 92.09, high solubility in water, freezing point at -38°C, and low toxicity (Fuller 2004). Glycerol is the most popular cryoprotectant for crypreservation of bovine sperm. The aplication of glycerol concentration dependent on species, in livestock animal 2-10% were applied. In bovine, 5-10% was applied but optimum concentration was 6-8% (Miller & Van Demark 1953). Glycerol applicating in one step was reported slightly toxic for sperm, then the glycerol was added in extender and given stepwise during the cooling process in a hour.

Action mechanism of intracellular cryoprotectant in maintaining cell during cryoproservation process was by replacing and filling the room left by water cell. By the low frozen point, glycerol was able to reduce cell damage caused by solution injury and ice crystallization (Fuller 2004; Andrabi 2007; Wowk 2007; Lemma 2011).

Intracellular cryoprotectant toxicity was highly depended on species response, concentration, temperature, and delivery technique (Wowk 2007). Glycerol were not toxic, but some research indicated toxicity glycerol in any species. The combination of intracelullar cryoprotectan glycerol with any extracelullar cryoprotectant can reduce the toxicity.

External cryoprotectant only worked on outside the cell with the same mechanism with the intracellular cryoprotectant. The extracellular cryoprotectant commonly is a carbohydrate or protein polimer. Alginate as encapsulation media component of sperm microencapsulation is expected may be act as extracellular cryoprotectant. Alginate is carbohydrate polimer with high molecule weight, not toxic, and can not penetrate spermatozoa cell membrane. There were no study of alginate action as extrecelullar cryoprotectant but carbohydrate polimer methyle cellulose was reported could be combined with glycerol to reduce glycerol negative effect against chicken's sperm fertility (Phillips et al. 1996). Alginate has also been applied in cryopreservation of prebiotic and fish. (Venugopal 2011; Pop et al. 2015).

This studys were conducted to determine the cryopropreservation technique of sperm microencapsulated using glycerol, to observe function of alginate "micro-encapsulation component " as extracellular cryoprotectant agent, whether process of microcapsulation affects sperm viability and to determine glycerol concentration at the cryopreservation of sperm microencapsulation. The final goal of this study was to increase artificial insemination efficiency.

## MATERIAL AND METHODS

Sperm cryopreservation was performed in two experiment. First experiment was to determine alginate and microencapsulation effect against sperm viability and second experiment was to determine glycerol concentration and equilibration time against sperm viability.

### Time and location

This study was conducted on June 2013-April 2014 at Laboratory of Reproduction Physiology, Indonesian Research Institute for Animal Production.

### Semen sample and semen quality evaluation

Three FH bulls (3-4 years old) were used and semen was collected twice a week using artificial vagina. Fresh semen quality observation was performed microscopically and macroscopically including color, consistentce, mass movement, live sperm percentage (%L), and intact acrosome ridge (%IAR).

Alive spermatozoa (%L) were observed using smear preparate by eosin-negrosin dye. Observation was

performed using light microscope with 100 x 10 magnifications. Dead spermatozoa would absorb the dye represent red color and the live were trasparant. Two hundred live and dead sperm were counted in 1 object glass and the live sperm were counted by following formulation:

$$\text{Live sperm (\%L)} = \frac{\text{Number live sperm}}{\text{Total sperm observed}} \times 100\%$$

Observation of %IAR was performed by observation sperm in NaCl formaline solution (0.01% w/v). One hundred sperm were observed under contrast phase microscope with 100 x 10 magnification using emersy oil. Percentage of IAR was calculated using the following formula:

$$\%\ \text{IAR} = \frac{\text{The number black hood sperm x 100\%}}{\text{Total sperm observed}}$$

Semen from 2 ejaculates (minimum mass movement was ++, %L was >80%, and concentration was more than 800 x 106/ml) were mixed proportionally to be used as sample. The pool semen was evaluated to obtain fresh semen quality data and used as repetition.

**Microcapsulation Sperm Preparation**

Twenty five ml encapsulation media (Table 1) was mixed with semen sample to get total concentration sperm 100 million/ml. Microgel formation was performed using microencapsulator Buchi B 390 (750 Hz, 550 volt). Droplets of media were collected into baker tube consisting 1.5% $CaCl_2$ and stirred using magnetic stirrer.

**Table 1.** Composition of Tris Citrate yolk-Alginat (TCEY-A) encapsulation media

| Chemical materials | Composition |
|---|---|
| Tris Hidroxymethil Aminomethan (g) | 3.027 |
| Citric Acid (g) | 1.675 |
| Fructose (g) | 1.25 |
| Streptomycin (mg) | 100 |
| Penicylin (IU) | 100000 |
| Glutation (mM) | 1 |
| Aquabides (ml) | 80 |
| Yolk (ml) | 20 |
| Alginate (g) | 1.5 |

Microgel formed was washed using physiological NaCl (3x) and weighed as 4 g as sample. Microcapsule membrane formation was performed using 1% chitosan (pH 5.5) for 15 minutes. The microcapsule was rinsed using physiological NaCl (3x) and liquid core performing was performed using 2% Na Citrate for 10 minutes. Evaluation of sperm viliability was conducted as soon as after microencapsulation process finished. The evaluation of sperm viability do using freezing media A (Table 3). It was used as initial data of sperm viability. Spermatozoa viliability variables observed were motility percentage (%M), %L, and %IAR.

Percentage of motility (%M) in the microencapsulation was observed by damaging microcapsule membrane using coverslip. Motility was observed under light microscope (100x10 magnification). The %L and %IAR were observed after microencapsule membrane were damaged using repetitive pipetting method (Kusumaningrum 2016).

**Research 1**

Research 1 was performed to determine the effect of alginate and microencapsulation process to viliability of sperm at each stage of freezing. Completely randomized design was used with 3 treatments and 6 repetitions. The treatments were T-I (semen sample in Tris citrate Egg Yolk_TCEY as control), T-II (semen sample in TCEY-A) and T-III (semen sample in TCEY-A followed by microcapsulation forming).

Semen sample was diluted using 3 diluent treatments to reach the final sperm cocentration 100 x $10^6$/ml. Evaluation of sperm viliability both of T-I, T-II, and T-III (%M, %L and %IAR) was performed after microencapsulation process (T-III) finished. Those data were used as initialy sperm viability data.

Feezing process both of T-I, T-II, and T-III was carried out by freezing standard of bovine’s sperm using Tris citric extender consisting of 20% yolk and 7% glycerol (Table 3) with equilibration time was 3 hours. Addition of freezing media (media A and B) was performed gradually started from room temperatur decrease until 5°C by using cooling machine.

Four ml sample semen (TI and TII) and four grams of microencapsule were add with 4 ml media A and B (Table 2) as following:

1. Addition of media A was performed after microcapsulation finished (2 ml)
2. Additional of media B was performed at 15 and 10ºC (2 ml)

The final concentration of sperm after that process was 50 x $10^6$/ml with glycerol concentration 7%. After temperature was at 5°C, evaluation of sperm viability (%M, %L and %IARAU) was carried out. Equlibration was carried out at 5ºC for 3 hours. In first 1 hour equilibration, semen was packed into medium straw (0.5 ml, 25x$10^6$/straw). Equlibration was continued in the straw until 3 hours equilibration.

Straw were freezed on 8 cm upper $N_2$ liquid for 10 minuts, then plung in $N_2$ liquid. Frozen sperm

evaluation was carried out after 1 week storage. The straw were thawed using warm water (37°C) for 30 seconds, and sperm vialbility (%M, %L, and %IAR) was evaluated. The datas were record as post-thawing data. Viliability data (initialy, 5°C, and post-thawing data) was analyzed by ANOVA and mean difference was tested using Duncan (SAS 9.1).

**Research 2**

This study was conducted by 2 x 2 factorial Completely Ramdomized Design with 6 repetitions. As first factor was glycerol concentration by 5 and 7% and the second factor was equilibration time by 3 and 4 hours.

2 ml of media A (Table 4) were added in 4 gram microcapsule, in room temperature. Temperature of suspension of semen was decreased using cooling mechine, temperature will decrease gradually until 5°C (±60 minutes). Two ml freezing media B (Table 4) was added stepwise at 15°C and 10°C to obtain sperm concentration by 50 x $10^6$/ml with glycerol concentration by 5 and 7%.

Equilibration was carried out in the cool top (5°C) for 3 and 4 hours. Packaging of sperm microencapsulated were performed using medium straw (0.5 ml, 25 x $10^6$/straw) at 1 hour after equilibration. Straws were seal using poliviline powder and equilibration was continued until 3 and 4 hours.

**Table 2**. Composistion of TCEY and TCEY-Alginate extender

| Chemical composition | TCEY | TCEY-Alginate |
|---|---|---|
| Tris Hydroxy Amino-methan (g) | 3.027 | 3.027 |
| Citric acid (g) | 1.675 | 1.675 |
| Fructose (g) | 1.25 | 1.25 |
| Streptomycin (mg) | 100 | 100 |
| Penicylin (IU) | 100000 | 100000 |
| Glutation (mM) | 1 | 1 |
| Aquabides (ml) | 80 | 80 |
| Yolk (ml) | 20 | 20 |
| Alginate (g) | - | 1.5 |

**Table 3.** Composittion of sperm freezing media (media A and B)

| Chemical composition | Media A | Media B |
|---|---|---|
| Tris Hydroxy Amino-methan (g) | 3.027 | 3.027 |
| Citric acid (g) | 1.675 | 1.675 |
| Fructose (g) | 1.25 | 1.25 |
| Streptomycin (mg) | 100 | 100 |
| Penicylin (IU) | 100000 | 100000 |
| Glutation (mM) | 1.0 | 1.0 |
| Aquabides (ml) | 80 | 80 |
| Yolk (ml) | 20 | 20 |
| Glycerol (ml) | 0 | 28 |

The freezing of straw were perfomed using the same methode before. The evaluation of post thawing were done 1 week after storage in $N_2$ liquid using same methode.

Sperm viability was analyzed using ANOVA. Difference between treatment was tested using Duncan and LSD if there was significant interaction between glycerol concentration and equilibration time (SAS 9.1).

## RESULT AND DISCUSSION

### Quality of fresh semen

Fresh semen used in this study was feasible to be used in sperma cryopreservation process. The semen has sufficient volume and concentration, average volume 5 ml, concentration 1160 x $10^9$, average percentage of live sperm 82.6% and 87.7% IAR.

### Research 1

Alginate and sperm microencapsulation process was affacting sperm vialbility at initial of cryopreservation process.

Sperm motility (%M) in media consisting alginate (T-1II) was lower (P<0.05) than control (T-I), meanwhile microencapsulation process caused %M on encapsulated T-III (55.00%) was lower (P<0.05) than unencapsulated. (66.71 and 60.00% for T-I and T-II, respectively).

The percentage of live sperm (%L) T-III (82%) was also lower (P<0.05) than non-encapsulated (90.14 and and 86.57 for T-I and T-II, respectively).

The percentage of IAR (%IAR) was not significantly different caused by alginate and microencapsulation process (Table 5).

### Viability of sperm at initialy of cryopreservation

Sperma vialbility (%M, %H dan %IAR) at the beginning of cryopreservation varied depending on process and media where spermatozoa stayed for a hour at the room temperature. Sperm viability inT-PI and T-II was in line with previous study conducted by Kusumaningrum et al. (2105), where %M in media TCEY-Alginate (T-II) lower (P<0.05) than TSKT (T-I), but %L and %IAR were not significant different.

TCEY-Alginate (T-II) lower (P<0.05) than TSKT (T-I), but %L and %IAR were not significant different. Microencapulation process (T-III) significant lower (P<0.05) than non-microencapsulation (T-I and T-II) at initial quality (%M and %H).

Microencapsulation process consisting of 3 stage and every stage is physically and chemicaly give stress to the sperm. At the first stage, formation of microgel: interaction sperm with media TCEY consisting 1.5% alginate which high osmilarity and viscosity decrese the sperm motility (Kusumaningrum et al. 2015). A contact between microencapsulation media with gel former media (1.5% $CaCl_2$) causing the sperm in immobilization phase. That contact also change sperm membrane condition, where Ca would stimulate change of distribution of lipid and composition pospolipid membrane as occurred on capacitation and acrosome reaction (Landim-Alvarenga et al. 2004).

In the second stage (alginate-chitosan membrane forming), sperm linked with chitosan solution with pH 5.5 and followed by washing process. Low pH might cause lost of spermatozoa membrane integrity (Bohloodi et al. 2012).

In the third stage, reaction of microcapsule-sodium citric solution causing sperm condition change from immotile into motile sperm inside the encapsulation

**Table 4**. Composition of freezing media A and B with different glycerol concentration

| Chemical composition | Media A | Media B | |
|---|---|---|---|
| | | Glycerol 5% | Glycerol 7% |
| Tris Hydroxy Amino-methan (g) | 3.027 | 3.027 | 3.027 |
| Citric acid (g) | 1.675 | 1.675 | 1.675 |
| Fructose (g) | 1.25 | 1.25 | 1.25 |
| Streptomycine (mg) | 100 | 100 | 100 |
| Penicylin (IU) | 100000 | 100000 | 100000 |
| Glutation (mM) | 1 | 1 | 1 |
| Aquabides (ml) | 80 | 80 | 80 |
| Yolk (ml) | 20 | 20 | 20 |
| Glicerol (ml) | 0 | 20 | 28 |

**Table 5**. Effect of alginate and sperm microencapsulation process to sperm vialibity

| Cryopreservation stage | Parameter | Treatment | | |
|---|---|---|---|---|
| | | T-I | T-II | T-III |
| Initialy | %M | 66.71±6.07[a] | 60.00±6.45[b] | 55.,71±3.45[c] |
| | %L | 90.14±5.70[a] | 86.57±6.55 [a] | 82.00±6.30[b] |
| | %IAR | 90.14±6.62[a] | 91.20 ±6.00[a] | 90.71 ±5.50[a] |
| 5°C | %M | 61.43±2.40[a] | 60.71± 1.89[a] | 55.00±5.00[b] |
| | %L | 89.14±9.70[a] | 86.86 ±8.15[a] | 82.86±4.42[a] |
| | %IAR | 90.86±3.02[a] | 90.86 ±2.61[a] | 85.71±5.72[b] |
| Post thawing | %M | 50.83±1.95[a] | 50.42 ±1.44[a] | 44.17±6.22[b] |
| | %L | 83.58±1.95[a] | 79.33 ±5.87[a] | 80.75±5.36[a] |
| | %IAR | 83.50±8.02[a] | 85.33 ±8.93[a] | 79.33±7.91[b] |

[a,b] Different letter in the same line shows significant difference ($P<0.05$)
Initialy = sperm viability observed simultanouslly before cryopreservation process
5°C = sperm viability at 5°C
Post thawing= sperm viability observed after thawing using water 37°C for 30 second

media. Rinsing process in every stage was other than to remove the substance excess, preventing occurrence of unexpected reactions, but also causing osmotic change of sperm caused by the dilution. It was reported that dilution caused ion concentration change other than causing dilution effect deceasing sperm viability (Hayden et al. 2015).

The microencapsulation process caused sperm quality decrease, especially the significant effect to %M and %L compared to %IAR. In spite of the %IAR in the initial cryopreservation was not significant which was affected by microencapsulation process, but this process caused acrosome ridge more sensitive to temperature change during cooling and freezing, so that decrease percentage of IAR decrease percentage on encapsulated sperm higher than non-encapsulated (Figure 1).

### Viability of sperm at 5°C

The decrease of temperature was significantly affect %M and %IAR, where the %M and %IAR in the sperm microencapsulation (%M = 55%, %IAR = 85.7%) was lower than non-encapsulated spermatozoa (average %M and %TAU of T-II and T-III was 61.07% and %IAR was 90.86% respectively). Spermatozoa viability at the initialy of microencapsulation process was be a main factor in this stage, meanwhile other factors associated with cooling process until reaching 5°C. Cold shock was not occurred in this study. Microencapsulation formation process as in T-III was indicated significant %IAR decrase compared to the T-I and T-II. The main factor causing spermatozoa quality decrease during temperature decrease to 5ºC was cold shock (Andrabi 2007) marked by cell metabolism decrease, membrane permeability increase, intracellular component losses, permanently motility decrease and increase of the number of dead cell (Lemma 2011).

Temperature decrease was significantly affect %M and %IAR on encapsulated spermatozoa (T-III), where %M and %IAR significantly lower ($P<0.05$) compared non-encapsulated. Decrease of %M and %IAR indicated an inhibition of microcapsule membrane and encapsulation media (high viscocity and osmolarity) causing glycerol effect to spermatozoa in microcapsule membrane became slow. Not like glycerol affecting sperm in the commonly freezing process, where glycerol was able to penetrate cell membrane in 2-5 minuts. It seemed that glycerol needed time to penetrate microcapsule membrane and encapsulation media which had high osmolarity and viscosity. To prevent decrease of %IAR during cooling process, modification of additional time before critical period of cold shock between 15-5°C was needed (Watson 2000).

### Viability of *post-thawing* sperm

Freezing and thawing processes was significantly affected motility and integrity of acrosome membrane of sperm, where %M and % IAER of encapsulated spermatozoa was lower ($P<0.05$) compared to non-encapsulated, meanwhile %L was not significantly affected by the freezing and thawing processes.

Quality of post-thawing of encapsulated spermatozoa, other than affected by freezing and thawing process, was also affected by preliminary quality and process of glycerol addition before reaching 5°C. Equilibration for 3 hours was success to improved quality of sperm microencapsulate (T-III), because

**Table 6**. Effect of glycerol and equilibrium time on post thawed sperm vialbility

| Parameter | Equilibration (hour) | Glycerol (%) | | Average |
|---|---|---|---|---|
| | | 5 | 7 | |
| %M | 3 | 42.14±7.56 | 43.86±6.36 | 42.50±6.72[a] |
| | 4 | 43.57±4.76 | 44.29±4.50 | 43.93±4.66[a] |
| | Average | 42.87 ±6.16[a] | 43.56±5.35[a] | |
| %L | 3 | 77.29±6.25 | 80.29±5.47 | 78.78±5.05[a] |
| | 4 | 77.57±4.58 | 79.86±5.43 | 78.72±4.97[a] |
| | Average | 77.43±6.72[a] | 80.71±5.26[a] | |
| %IAR | 3 | 76.86±8.36 | 78.14±6.26 | 77.50±7.75[a] |
| | 4 | 78.29±7.70 | 78.86±8.09 | 78.57±6.69[a] |
| | Average | 77.57±7.12[a] | 78.50±7.59[a] | |

[ab]Different superscrip in the same column and lines shows significant difference (P<0.05)

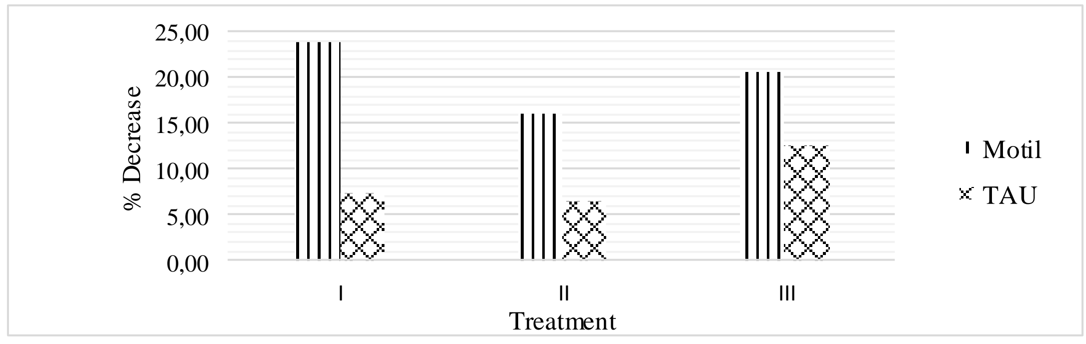


**Figure 1.** Percentage of decrease of %M and %IAR due to alginate (II) and microcapsulation process (III) compared with control (I)

glycerol had proven protective effect to sperm inside the microencapsulate membrane. Glycerol was able to protect sperm cell in the microcapsule, so that during freezing and thawing process, %IAR of T-III only decreased by 6.38%, meanwhile decrease of %IAR T-II was 5.53% and the control (T-I) decreased by 7.36% (Table 6).

The percentage of decreasing %M and %IAR counted to determine contribution of alginate and process of microencapsulation to %M and %IAR of the post-thawing. The decreasing of percentage of %M and %IAR at the initial of freezing until post-thawing were presented in the Figure 1.

Percentage of decrease of post-thawing quality in the T-II (15.97% for %M and 6.44% for %IAR) was lower than T-I (23.8% for %M and 7.73% for %IAR). It indicated there was alginate role considering that T-II consisted of 0.75% alginate in to total media added.

Decrease of sperm quality in T-III higher than T-II (20.71% for %M and 12.55 for %IAR) showed that contribution of microcapsulation process against %M and %IAR post-thawing of microencapsulation sperm. Lower percentage decrease of %M dan %IAR in T-III compared to T-I also showed alginate effect.

Alginate, carbohydrate polimer with high molecule weight, migh play role as extracellular cryoprotectant due to its ability to protect sperm then decrease of %M and %IAR from T-II lower than control (T-I). Alginate protection mechanism was extracellular ptotection mechanism, because alginate might not able to penetrate cell membrane. Action mechanism of alginate in this study has not been known.

Alginate use as extracellular cryoprotectant in sperm cryopreservation has not been reported before. It was reported that carboxyl cellulose (similar with alginate) might be used as extracellular cryoprotectant at chicken's semen cryopreservation (Phillips et al. 1996). Alginate was also used in cryopreservation of fish to prevent protein denaturation (Venugopal 2011) and cryopreservation of probiotik (Pop et al. 2015). Futher study of role and concentration of alginate as extracellular cryprotectant in sperm cryopreservation process was needed.

**Research 2**

There was no significant interaction between glycerol concentration with equilibrium time. Decrease

of glycerol concentration from 7% into 5% did not significantly affect viliability of sperm microencapsulation both in %M, %L, and %IAR (Table 6).

The process of cryopreservation of sperm microencapsulation were done using conventional process of cryopreservation sperm. Bovine sperm cryopreservation was commonly performed using combined TCEY (20% yolk) extender and glycerol at 7% concentration. Glycerol added during cooling process (Muino et al. 2007) and followed by equlibration at 4-5°C (Leita et al. 2010) for 2-18 hours to obtain optimal fertility (Muino et al. 2007).

Equilibration period length was feared to be a cause of fertility decrease caused by sperm aging process, so that shortening the equilibration period was an option. Effect of equilibration time to sperm motility, membrane integrity, and mitocondria function were still being debated. However, previous study showed that equilibration time by 4-6 hours produced the best motility, meanwhile study using CASA against progresive of motility and integrity of plasm and acrosome mebrane showed 4 hours equilibrium was the best time for bovine spermatozoa cryopreservation process (Leita at al. 2010).

The duration of equilibration at cryopreservation of microencapsulated sperm, showed that 4 hours equilibration had no significant different (on %M, %L, and %IAR) compared to the 3 hours equilibrium. There is no previous study about optimal equilibrium time in cryopreservation of microencapsulation of bovine sperm.

The effect of glycerol concentration (7 vs 5%) at freezing of microencapsulation sperm freezing were not significantly, so that the 5% glycerol might be used to reduce negative effect of glycerol. Glycerol concentration 7% l was optimal concentration to be used in bovine spermatozoa cryopreservation (Miller & Van Demark 1953). Glycerol concentration 7% is optimal concentration to be used in bovine sperm cryopreservation (Miller & Van Demark 1953). In cryopreservation of sperm microencapsulation, the concentration of glycerol might be decreased into 5%. The decrease concebtration from 7% into 5% did not cause harm effect to %M, %L and %IAR. This was caused by alginate role which was a part of encapsulation media. Alginate may act as extracellular cryoprotectants such as the role of the cellulose in the cryopreservation of poultry semen (Phillips et al. 1996). Further study on effect of alginate concentration decrease from 7% into 5% by considering alginate as extracellular cryoprotectant agent needed to be developed.

## CONCLUSION

Cryopreservation for microencapsulated of bovine sperm could be carried out using standard protocol cryopreservation bovine sperm. Glycerol can be aplicated in concentration 5 or 7% with time of equilibration 3-4 hour.

Microencapsulation processes altered the viability of sperm. Alginate might play a role as extracellular cryoprotectant due to its ability to prevent %M and %IAR decreasing along the freezing of microencapsulation sperm, due to alginate can be reduce glycerol concentration at 5%. It was suggested to study alginate utilization as extracellular cryoprotectant.

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# Gas Production and Rumen Fermentation Characteristics of Buffalo Diets Containing By-Product from Some Sorghum Varieties

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## ABSTRAK

Sugoro I, Wiryawan KG, Astuti DA, Wahyono T. 2015. Karakteristik produksi gas dan fermentasi rumen pada ransum kerbau yang mengandung hasil samping dari beberapa varietas tanaman sorgum. JITV 20(4): 242-249. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1241

Sumber serat yang berasal dari sorgum adalah salah satu bahan pakan potensial untuk kerbau. Sorgum Samurai 1 dan Samurai 2 merupakan varietas sorgum yang berasal dari pemuliaan mutasi radiasi indukan sorgum varietas Pahat. Tujuan dari penelitian ini adalah untuk membandingkan secara *in vitro* ransum kerbau yang mengandung 50% sumber serat dari jerami sorgum Samurai 2 atau bagas sorgum Samurai 1 dibandingkan dengan ransum yang mengandung 50% jerami sorgum Pahat. Penelitian ini menggunakan Rancangan Acak Lengkap enam perlakuan dengan tiga ulangan. Pengambilan cairan rumen kerbau dalam waktu yang berbeda berperan sebagai ulangan. Enam pakan perlakuan terdiri dari P1 (50% jerami sorgum Pahat + 50% konsentrat), P2 (50% silase jerami sorgum Pahat + 50% konsentrat), P3 (50% jerami sorgum Samurai 2 + 50% konsentrat), P4 (50% silase jerami sorgum Samurai 2 + 50% konsentrat), P5 (50% bagas sorgum Samurai 1 + 50% konsentrat) dan P6 (50% silase bagas sorgum Samurai 1 + 50% konsentrat). Sebanyak 200 mg bahan kering (BK) ransum diinkubasi dalam 30 ml medium cairan rumen-buffer selama 48 jam. Peubah yang diamati adalah produksi gas total, konsentrasi $CH_4$ dan karakteristik fermentasi rumen. Hasil penelitian menunjukkan bahwa P2 dan P4 menghasilkan produksi gas tertinggi ($P<0,05$) berturut-turut 60,99 dan 60,86 ml/200 mg BK. Perlakuan P1, P2 dan P4 menghasilkan konsentrasi $CH_4$ terendah ($P<0,05$) dengan nilai berturut-turut 10,57; 10,90; dan 9,82% dari total gas. Perlakuan P4 menghasilkan VFA total, degradasi Bahan Kering (BK) dan degradasi Bahan Organik (BO) dengan nilai berturut-turut 109,83 mM; 62,93; dan 59,97%. Konsentrasi amonia ($NH_3$) tidak berbeda nyata antar perlakuan. Kesimpulan dari penelitian ini adalah sumber serat silase jerami sorgum Samurai 2 lebih baik dibandingkan jerami sorgum Pahat dan bagas sorgum Samurai 1 dalam ransum kerbau.

**Kata Kunci:** Fermentasi Rumen, Konsentrasi CH4, Kerbau, Sorgum

## ABSTRACT

Sugoro I, Wiryawan KG, Astuti DA, Wahyono T. 2015. Gas production and rumen fermentation characteristics of buffalo diets containing by-product from some sorghum varieties. JITV 20(4): 242-249. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1241

Sorghum is one of potencial fibre sources as buffalo feed. Quality of sorghum could be increased by irradiation mutation breeding. Samurai 1 and Samurai 2 were products of the irradiation mutation breeding of Pahat. This study was conducted to compare buffalo diets containing Samurai 2 sorghum straw and Samurai 1 bagasse sorghum compared with Pahat sorghum straw using in vitro study. Completely randomized design with 6 treatments and 3 replications was applied in this experiment. The treatment diets were P1 (50% Pahat sorghum straw + 50% concentrate), P2 (50% Pahat sorghum straw silage + 50% concentrate), P3 (50% Samurai 2 sorghum straw + 50% concentrate), P4 (50% Samurai 2 sorghum straw silage + 50% concentrate), P5 (50% Samurai 1 sorghum bagasse + 50% concentrate) and P6 (50% Samurai 1 sorghum bagasse silage + 50% concentrate). The 200 mg DM samples of diets were incubated in 30 ml rumen-buffer fluid for 48 hours. Variables measured were total gas production, $CH_4$ production and rumen fermentation characteristics. Results showed that P2 and P4 produce the highest of gas production ($P<0.05$) with 60.99 and 60.86 ml/200 mg dry matter respectively. Treatments of P1, P2 and P4 produced the lowest $CH_4$ concentration ($P<0.05$) with 10.57, 10.90 and 9.82% of total gas, respectively. The P4 produced the highest total volatile fatty acids (VFA), dry matter degradability and organic matter degradability with 109.83 mM, 62.93% and 59.97% respectively, meanwhile ammonia ($NH_3$) concentration was not significantly different. The conclusion showed that straw silage of Samurai 2 was comparable to the Pahat sorghum straw and Samurai 1 bagasse sorghum as buffalo diet.

**Key Words:** Buffalo, CH4 Concentration, Rumen Fermentation Characteristics, Sorghum

## INTRODUCTION

Utilization of sorghum for forage will strongly support livestock management in marginal area. Sorghum is a multifunctional crop producing food, feed, bioethanol and another industrial material (Sirappa 2003). As a fibre sources, sorghum may be fed in form of fresh straw or silage. Silage processing was

aimed for preservation and nutrients retaining (Colombo et al. 2007). Combined sorghum straw and concentrate diet had equal quality and may replace combined corn straw and concentrate diet. Fibre of sorghum crop may be also obtained from sorghum bagasse, which is a processing waste of bioethanol made of sorghum. Combined baggase and concentrate (50 : 50) was able to increase milk fat level (7.61%) of Murrah buffalo and to maintain milk yield more than 5 kg/day (Seshaiah et al. 2013).

Several sorghum varieties are suitable to be developed in dry land, ideal for food industrial, suitable for bioethanol and have good palatability. Those varieties are Pahat, Samurai 1 and Samurai 2. Those varieties were breeding result by gamma irradiation conducted by National Nuclear Energy Agency (Human 2013). Advantages of Pahat are high seed productivity (5 t/ha), low tannin (0.012%), semi-short rod (158 cm), and a multifunctional food and feed resources. Advantages of Samurai 1 are high seed productivity (7.5 t/ha), high rod sugar level (12-18%), and high biomass appropriate for bioethanol substance. Meanwhile, advantages of Samurai 2 are high biomass productivity (47 t/ha), leaf rust diseases (*Hemileia vastatrix*) and rotten midrib (Sihono et al. 2013). Pahat is a sorghum breed producing Samurai 1 and Samurai 2 varieties.

By-products such as straw and bagasse from those varieties may be used as fibre resources for buffalo diet. Those fibre sources fed in form of silage or fresh. Nutrient value of the third varieties needs to be compared considering that the Samurai 1 and samurai 2 are a derivative of Pahat. Comparation of nutrient value of the silage and fresh fibre resources needs to be conducted to determine nutrient value change occurred due to the breeding process. This study was aimed to compare buffalo diet consisting of 50% fibre source from sorghum straw Samurai 2 or bagass Samurai 1(as mutants) with diet consisting of 50% sorghum straw Pahat (as breed).

## MATERIALS AND METHODS

### Materials preparation

Forages used in this study were sorghum straw Pahat, Samurai 1, Samurai 2, silage of sorghum straw Pahat and Samurai 2, bagass Samurai 1, silage of bagass Samurai 1and concentrate. Pahat and Samurai 1 used were the leaves and. Whereras, the bagass Samurai 1 used was squeezed-stems. Pahat and Samurai 2 were harvested in 80 days old. Samurai 1 was harvested in 100 days old as bioethanol raw material. Silage of sorghum straw and bagass were withered and chopped by ±2 cm. the chopped silage was putted in plastic drum silo with capacity 20 kg and incubated anaerobically for 21 days. Additive was not used during the silage processing. After that, study materials were dried in oven 60°C, grilled and filtered by 1 mm filterer.

### Experimental design

This study used Complete Randomize Design with 3 replications with following model:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where:
Yij = the ith treatment observation and jth replication,
M = general mean,
Ai = effect of ith treatment,
Eij = random affect of Ith factor and jth replication.

Data obtained will be analyzed using analysis of variance (ANOVA) and followed by Duncan further test (Mattjik & Sumertajaya 2006) helped by SPSS 16.0.

Buffalo's rumen collection during diferent incubation played as replication. This study used 6 treatments presented in Table 2. Diet was formulated based on nutrient requirement of lactation buffalo (CP 8%) (Parakkasi 1999).

### Hohenheim gas test (Menke et al. 1979)

Two hundred ±10 mg of sample was putted into syringe with vaseline smeared-piston. The sample was incubated according to Menke et al. (1979) for 48 hours into 1241.76 ml Mc Dougall fluid (Krishnamoorthy 2001) and 650 ml rumen fluid. Rumen fluid was obtained from fistulated-buffalo routinely fed by grass and concentrate with ratio by 50 : 50 in DM. Rumen collection was carried out in the morning before fed. The liquor was filtered by 4 layer-gauze. Incubation was conducted in waterbath 39ºC. Thirty ml media liquor was putted into each syringe. Piston in the syringe was pressed until no empty cavity. Initial volume before sample incubated was recorded. Variables observed were total gas production, CH4 production, kinetic gas, ammonia (NH3), volatile fatty acid (VFA), dry material (DM) degradation, and organic material (OM) degradation.

### Measurement of rumen fermentation variables

Variables observed were total gas production, $CH_4$ production and rumen fermentation products such as $NH_3$, VFA, dry material (DM) degradation, and organic material (OM).

Gas production was recorded on 2, 4, 6, 8, 10, 12, 24 and 48 hour. Gas production reading was quickly conducted to minimize temperature change. This measurement was according to Krishnamoorthy (2001).

**Table 1**. Composition of experimental diet based on DM with fibre source and concentrate ratio by 50 : 50

| Diet Raw Materials | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | P4 | P5 | P6 |
| Soybean meal | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 |
| Pollard | 5 | 5 | 5 | 5 | 5 | 5 |
| Onggok (cassava waste) | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 | 14.5 |
| Rice bran | 14.25 | 14.25 | 14.25 | 14.25 | 14.25 | 14.25 |
| Soy ketchup pulp | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 |
| Lacta mineral | 1 | 1 | 1 | 1 | 1 | 1 |
| Urea | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 |
| Salt | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Lime | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Molases | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Straw sorghum Pahat | 50 | - | - | - | - | - |
| Straw sorghum silage Pahat | - | 50 | - | - | - | - |
| Straw sorghum Samurai 2 | - | - | 50 | - | - | - |
| Straw sorghum silage Samurai 2 | - | - | - | 50 | - | - |
| Bagass sorghum Samurai 1 | - | - | - | - | 50 | - |
| Bagass sorghum silage Samurai 1 | - | - | - | - | - | 50 |

Kinetic gas was measured by exponential method of Ørskov & McDonald (1979) p = a + b (1-e-ct). a and b constants were soluble and insoluble fraction but may be degradable, respectively. The c constant was constantly fraction rate per t unit time. Calculation of a, b, and c used fitcurve Naway® software.

Measurement of $CH_4$ was carried out after determination of $CH_4$ concentration in total gas from fermentation of each syringe. Measurement of $CH_4$ concentration conducted using MRU gas Analyzer®. $CH_4$ concentration measurement was conducted to incubation result of the 48$^{th}$ hour. Readed value on MRU gas Analyzer® was percentation of $CH_4$ stored in the syringe. Variables observed were $CH_4$ concentration and $CH_4$ production of buffalo diet in every 200 mg digested organic matter. $NH_3$ measurement was carried out by Conway microdifusion method in GLP (1966). VFA measurement was carried out by steam destilation method (Warner 1964). DM and OM measurements were carried out according to measurement of Blümmel et al. (1997).

## RESULT AND DISCUSSION

### Total gas production

Measurement result of the total gas production was presented in Table 3. The total gas production between treatments was significantly no different on 2$^{nd}$ and 4$^{th}$ hour incubation. Difference began to show in 6$^{th}$ hour incubation. The highest total gas production was on 8$^{th}$-12$^{th}$ incubation produced by P6. On the 24$^{th}$ hour incubation, the highest total gas production was produced by P4, P6, and P2 ($P<0.05$). On the 48$^{th}$ hour incubation was produced by P4 and P2. Gas production gradualy increased with increasing the incubation time. $CO_2$ and $CH_4$ produced in this method derived from directly and undirectly substrate fermentation through VFA buffering mechanism, that is $CO_2$ which was released from buffer bicarbonate produced during fermentation process (Getachew et al. 1998; Jayanegara et al. 2009).

Gas profile dynamic was related to nutrient content difference in the six diets (Table 2). P1, P3, and P4 treatments had low gas production rate in the early incubation hours due to high crude fibre (CF) content, in the contrary with P2 and P4 that produced the highest gas in the early hour incubation due to the lowest CF content. High fibre in diet might cause decrease on rumen microbial activity on less than 24 hours of diet incubation (Kumar et al. 2007), however gas production rate from fermentation by microbe was higher on 48 hours of incubation. This was caused by rumen microbial started to digest the fiber after 24 hours incubation.

Coefficient of gas production kinetic was calculated by exponential model of Ørskov & McDonald (1979), where maximum gas production (a+b) and gas production rate (c) presented in Table 3. The maximum gas production (a+b) was significantly different

**Table 2**. Composition of experimental diet nutrient basen on DM with fibre source and concentrate ratio by 50 : 50

| Nutrient composition (%) | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | P4 | P5 | P6 |
| Dry matter | 90.37 | 89.51 | 90.36 | 88.08 | 90.33 | 89.22 |
| Organic matter | 86.75 | 85.34 | 85.32 | 85.34 | 84.33 | 87.48 |
| Crude protein | 9.04 | 12.15 | 11.04 | 11.86 | 8.57 | 8.25 |
| Crude fiber | 26.06 | 22.67 | 24.80 | 26.26 | 23.26 | 19.66 |
| Crude fat | 2.71 | 1.14 | 1.40 | 1.38 | 1.72 | 2.06 |
| Free nitrogen extract | 48.94 | 49.38 | 48.08 | 45.84 | 50.78 | 57.51 |

**Table 3**. Production of total gas and kinetic gas in vitro of buffalo diet containing of sorghum during incubation for 2-48 hours (ml/200mg bk)

| Treatment | Incubation time (hour) | | | | | | | | Kinetic gas | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 24 | 48 | a+b | c |
| P1 | 7.04 | 11.90 | $16.10^{b}$ | $19.37^{b}$ | $23.36^{b}$ | $26.25^{c}$ | $39.31^{c}$ | $52.77^{c}$ | $59.22^{ab}$ | 0.045 |
| P2 | 9.38 | 14.97 | $19.69^{a}$ | $23.27^{ab}$ | $26.84^{ab}$ | $30.42^{ab}$ | $42.96^{ab}$ | $56.19^{a}$ | $60.99^{a}$ | 0.050 |
| P3 | 7.50 | 12.14 | $16.54^{ab}$ | $20.54^{ab}$ | $23.61^{b}$ | $27.60^{bc}$ | $40.05^{c}$ | $52.67^{c}$ | $57.92^{b}$ | 0.049 |
| P4 | 7.77 | 13.90 | $18.61^{ab}$ | $22.38^{ab}$ | $25.92^{ab}$ | $30.16^{ab}$ | $44.29^{a}$ | $56.31^{a}$ | $60.86^{a}$ | 0.053 |
| P5 | 8.27 | 14.05 | $18.74^{ab}$ | $22.55^{ab}$ | $26.98^{ab}$ | $30.18^{ab}$ | $42.14^{b}$ | $54.04^{bc}$ | $57.21^{b}$ | 0.057 |
| P6 | 7.61 | 14.06 | $19.45^{ab}$ | $23.93^{a}$ | $28.02^{a}$ | $31.70^{a}$ | $43.64^{ab}$ | $54.86^{ab}$ | $56.99^{b}$ | 0.065 |
| SEM | 0.414 | 0.434 | 0.479 | 0.563 | 0.588 | 0.577 | 0.476 | 0.403 | 0.492 | 0.002 |

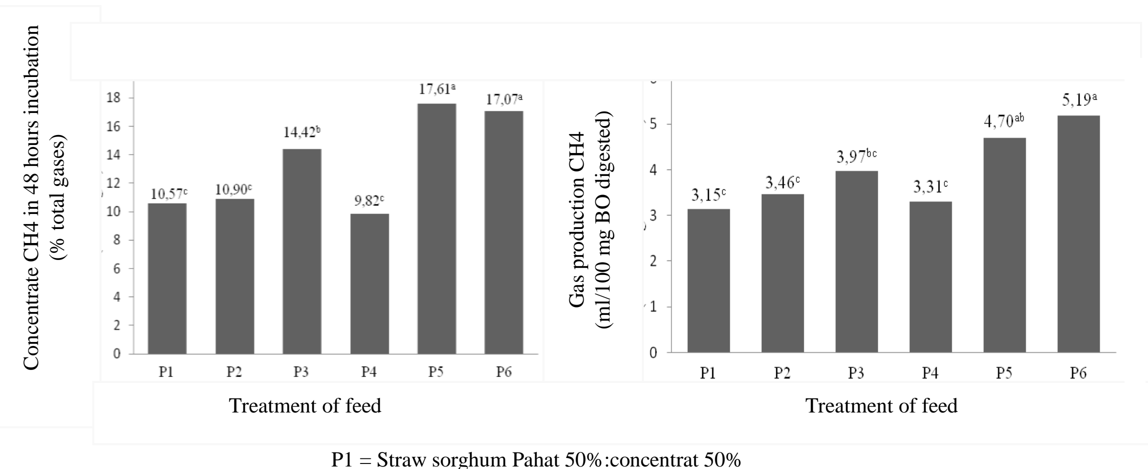
P1 = Straw sorghum Pahat 50%:concentrat 50%
P2 = Straw sorghum silage Pahat 50%:concentrat 50%
P3 = Straw sorghum Samurai 2 50%:concentrat 50%
P4 = Straw sorghum silage Samurai 2 50%:concentrat 50%
P5 = Bagass sorghum Samurai 1 50%:concentrat 50%
P6 = Silage bagass sorghum Samurai 1 50%:concentrat 50%
Different superscript in the same column shows significantly difference (P<0.05);
a+b = Maximum gas production;
c= Gas production rate;
SEM = *Standard Error Mean*

(P<0.05) between treatments. Gas production rate was not significantly different in all treatments. The highest maximum gas production was produced by P2 and P4 and has the same trend with CP content of diet (Table 2). P2 and P4 consisted of high CP and tend to produce higher maximum gas production. Different with Luna et al. (2013) who reported that CP content had no correlation with maximum gas production. Protein fermentation would produce aminia affecting buffer bicarbonate balance through ion $H^+$ neutralization mechanism without releasing $CO_2$ (Cone & Van Gelder 1999; Salem et al. 2013). This difference was caused by diet substrate difference in the treatment. Combination of sorghum and concentrate in this study might maximize CP quality of diet for microbial fermentation. Non Protein Nitrogen (NPN) obtained from urea in the concentrate was able to be optimized for microbial protein synthesis. Optimal microbial performance affected increase of maximum gas production.

**$CH_4$ production**

Total gas production increasing was followed by $CH_4$ production. Higher total gas production caused high $CH_4$ production. $CH_4$ variable was used to measure $CH_4$ emition decrease level. $CH_4$ concentration of buffalo diet from 48 hour incubation and $CH_4$ production in every 100 mg organic material were presented in Figure 1. The lowest $CH_4$ concentration was produced by P4 and had no significant difference with P1 and P2. The lowest CH4 production per unit digested OM was produced by P1 and had no significant difference with P4, P2, and P3. The highest $CH_4$ concentration was produced by P5 with higher concentration by 66.60% (P<0.05) compared with P1. $CH_4$ concentration in P6 was also higher by 61.49% (P<0.05) than P1.

High $CH_4$ concentration and production per unit OM digested in P5 and P6 were affected by low CP content. Widiawati et al. (2007) reported that higher




P1 = Straw sorghum Pahat 50%:concentrat 50%
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P3 = Straw sorghum Samurai 2 50%:concentrat 50%
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P5 = Bagass sorghum Samurai 1 50%:concentrat 50%
P6 = Silage bagass sorghum Samurai 1 50%:concentrat 50%
Different superscript in the same column shows significantly difference (P<0.05)

**Figure 1**. Concentration of CH4 of buffalo diet from 48 hour incubation (left) and CH4 production of the diet in every 100 mg organic matter digested (right)

content and good quality of CP might produce lower $CH_4$. Protein degradation would produce NH4 combined with $CO_2$ to produce lower $CH_4$ (Getachew et al. 1998). Negative effect of fermentative digestion in the rumen was a lot of $CH_4$ wasted. Energy losses in feed become $CH_4$ reached 7% in ruminant (Kamal 1994). Baker (1999) reported that $CH_4$ production indicated a lot of energy losses in form of gas showing low feed efficiency. Low $CH_4$ concentration in P1, P2, and P4 showed the third diets were able to increase efficiency of rumen fermentation.

High $CH_4$ concentration in P5 and P6 might also be caused by fibre resources used was bagass sorghum harvested in 100 days old. P1, P2, P3, and P4 used sorghum fibre source harvested in 80 days old, so that $CH_4$ concentration produced was lower. Difference of harvest age affected dissolved and structured carbohydrate content. The dissolved carbohydrate would decrease, whereas structured carbohydrate would increase as well as crop age. Structured carbohydrate would be more difficult to be degraded than dissolved carbohydrate (Van Soest 1994). Widiawati et al. (2007) reported that high cellwall fraction produced high portion of acetat acid and $CH_4$. In Shahbazi (2008), it was reported that cellwall fraction was slowly fermented due to cellulose which was acociated in the lignin matrix. Besides, silage treatment caused the structured carbohydrate getting less due to degradation process carried out by microba during incubation (Yahaya et al. 2002).

It was suspected that there were cellwall fraction in the fibre source in the P5 and P6. It could be seen in low maximum gas production (a+b coefficient) in the P5 and P6 (Table 3). High cellwall fraction produced high $CH_4$. Utilization of sorghum fibre resource Pahat and Samurai 2 in P1, P2, P3, and P4 harvested in 80 days old had low concentration $CH_4$ and $CH_4$ production per unit OM digested compared to the P5 and P6. P2 and P4 diet, which was silage, was able to decrease $CH_4$ and $CH_4$ production per unit digested OM, but not in P6 (bagass silage). This was suspected occurred because bagass consisted of higher structured carbohydrate due to the squeeze processing. Decrease of $CH_4$ production might decrease wasted feed energy losses, so that increasing efficiency of feed utilization (Widiawati et al. 2007).

## Characteristic of rumen fermentation

Characteristics of rumen fermentation observed were $NH_3$, VFA, DMD, and OMD produced after 48 hour incubation (Table 4). $NH_3$ was not significantly different between treatments. VFA, DMD, and OMD showed significant different ($P<0.05$) between treatments. P4 showed highest in those fourth variables. $NH_3$ concentration between treatments showed no significant different, even though P4 tended to produce high $NH_3$ concentration (39.74 mg/100 ml). Not significant different between treatments was caused by percentage of NPN (urea) resources use in identic diet

**Table 4.** Characteristic of rumen fermentation *In Vitro* of buffalo diet based on sorghum in 48 hour incubation

| Variable | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | P4 | P5 | P6 | SEM |
| NH3 (mg/100ml) | 36.79 | 34.94 | 33.85 | 39.74 | 34.23 | 33.97 | 0.942 |
| VFA total (mM) | 91.32$^{b}$ | 86.38$^{b}$ | 91.32$^{b}$ | 109.83$^{a}$ | 92.55$^{b}$ | 92.55$^{b}$ | 2.321 |
| DMD (%) | 57.57$^{ab}$ | 60.59$^{ab}$ | 55.90$^{b}$ | 62.93$^{a}$ | 54.73$^{b}$ | 58.59$^{ab}$ | 0.912 |
| OMD (%) | 56.44$^{ab}$ | 56.42$^{ab}$ | 52.44$^{bc}$ | 59.97$^{a}$ | 49.31$^{c}$ | 55.57$^{abc}$ | 1.073 |

P1 = Straw sorghum Pahat 50%:concentrat 50%)
P2 = Straw sorghum silage Pahat 50%:concentrat 50%)
P3 = Straw sorghum Samurai 2 50%:concentrat 50%)
P4 = Straw sorghum silage Samurai 2 50%:concentrat 50%)
P5 = Bagass sorghum Samurai 1 50%:concentrat 50%)
P6 = Silage bagass sorghum Samurai 1 50%:concentrat 50%)
Different superscript in the same column shows significantly difference (P<0.05); SEM = *standard error mean*

(Table 1). Kang & Wanapat (2013) reported that buffalo rumen microbial had high efficiency in NPN use to synthesize microbial protein compared to another feed stuff. Amount of protein of diet was a factor affecting $NH_3$ production (McDonald et al. 2002). Different result in this study showed CP content of diet had no affect $NH_3$ concentration. This was suspected due to variated degradation level from different protein concentration feed resources.

NH3 showed high concentration around 33.85-39.74 mg/100 ml in all treatments. Concentrate content in treatment affected high ammonia production due to increase of sample protein content. This result was different with optimal concentration (5 mg/100 ml) for microbial fermentation in closed culture system and depended on feed fermentability level (Wanapat & Rowlison 2007; Wanapat et al. 2013). Optimal $NH_3$ concentration in the rumen of swamp buffalo was 14 mg/100 ml (Wanapat & Pimpa 1999).

The highest total VFA concentration was produced by P4 compared to another five treatments (P<0.05). Leng and Leonard who were cited by Pamungkas et al. (2006) reported that fermentation rate was correlated with VFA concentration, so that VFA concentration change was a reflection of increase of rumen microbial population. One of factors affecting high production of total VFA in P4 was high concentration of $NH_3$. This was an indicator of increase of microbial protein synthesis to increase rumen microbial population. Gas production rate (c), which tended to high in Table 3, also reflected a high VFA concentration as final product. The P4, P5, and P6 produced high total VFA as well as gas production rate.

Total VFA concentration in rumen-fistulated swamp buffalo fed by sorghum as single diet was 53.5 mM. Buffalo rumen fed by fermented rice straw and concentrate produced total VFA concentration by 44.8, 48.9, and 55.9 mM, respectively (Chanthakhoun & Wanapat 2012). Su-jiang et al. (2016) reported that total VFA from in vitro incubation in sweet sorghum silage and sorghum silage was 35.0 and 27.54 mM, respectively. Result in this study showed total VFA concentration around 86.38-109.83 mM, respectively. This higher result was caused by feed substrate difference. Fibre source from sorghum was more optimal to support rumen microbial performance if it was combined with concentrate in the diet.

The highest DMD and OMD was produced by P4, but were no significantly different than P1, P2, and P6. P4 produced higher DMD and OMD than P3 and P5 (P<0.05). Diet with silage as fibre source (P2, P4, and P6) tended to produce higher OMD than P1, P3, and P5. This respon was caused by structured carbohydrate content in the fibre source have been fermented by microb in the silage process. This caused faster degradation of DM. Silage treatment might increase degradation value of OM due to microflora activity during fermentation. The activity was effect of cellulase extra-curricular enzyme cutting cellulose bind in the silage substance (Jancik et al. 2011). OMD value, numericaly, was proportional to $NH_3$ concentration (Table 4) and maximum gas production value (a+b) (Table 3). High maximum gas production reflected organic material degradation level in diet. This proved that optimal fermentation level during 48 hour incubation was produced by P1, P2, and P4. From the production of total VFA, DMD, OMD, and low production of $CH_4$, P4 was better than other treatments. Sorghum silage Samurai 2 might be used to substitute fibre source from sorghum straw and sorghum bagass.

## CONCLUSION

Samurai 2 was better than Pahat and Samurai 1 for buffalo diet. This was presented by high production of gas, total VFA, and Dry Matter Degradation. Diet containing of Samurai 2 also produced low $CH_4$ *in vitro*. It needed further *in vitro* test to strengthen evaluation study of this buffalo diet.

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# Role of Sweet Orange (*Citrus sinensis*) Waste in Lowering the Meat Cholesterol and Fat of Padjadjaran Sheep

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**ABSTRAK**

Adriani L, Hernawan E, Hidayat U. 2015. Peran limbah jeruk manis (*Citrus sinensis*) dalam menurunkan kadar kolesterol dan lemak daging pada domba Padjadjaran. JITV 20(4): 250-256. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1250

Penelitian bertujuan untuk mengevaluasi peran limbah jeruk manis dalam menurunkan kadar kolesterol dan lemak daging pada domba Padjadjaran. Dua puluh ekor domba jantan dengan bobot badan 29,66±2,74 kg dan koefisien variasi <10% dilibatkan dalam penelitian dengan menggunakan Rancangan Acak Lengkap (RAL). Perlakuan terdiri atas 4 taraf limbah jeruk manis dalam ransum masing-masing T1 (0%), T2 (7%), T3 (12%) dan T4 (17%), perlakuan diulang 5 kali. Penelitian dilakukan selama 5 minggu, dengan peubah yang diamati meliputi konsumsi ransum diukur setiap hari, pertambahan bobot badan harian dan konsumsi air minum diberikan secara *ad libitum*, kolesterol dan trigliserida darah diukur pada akhir penelitian. Sampel daging diambil secara acak dari hasil pemotongan tiga (3) domba per ulangan. Hasil pengamatan menunjukkan penurunan kadar kolesterol daging tertinggi diperoleh pada T2, yaitu bagian paha 9,43±0,04 mg/mg dan dada 9,71±0,04 mg/mg, sementara penurunan lemak daging diperoleh pada perlakuan T4, yaitu bagian paha dan dada secara berurutan 9,70±3,98 ug/mg dan 10,48±1,85 ug/mg. pH rumen pada tidak menunjukkan perbedaan yang nyata (P>0,05) pada semua pelakuan, dengan kisaran nilai dari 6.80±0,01 - 6,50±0,02 µg/mg. Kesimpulan, limbah jeruk manis (*Citrus sinensis*) mampu berperan menurunkan kadar kolesterol dan lemak dan mempertahankan ekologi rumen dalam hal tingkat pH rumen Domba Padjadjaran.

**Kata Kunci**: Limbah Jeruk Manis, Kolesterol Daging, Lemak Daging, Domba Padjadjaran

**ABSTRACT**

Adriani L, Hernawan E, Hidayat U. 2015. Role of sweet orange (*Citrus sinensis*) waste in lowering the meat cholesterol and fat of Padjadjaran sheep. JITV 20(4): 250-256. DOI: http://dx.doi.org/10.14334/jitv.v21i1.1250

This research is aimed to evaluate sweet orange's role in lowering meat cholesterol and fat in Padjadjaran sheep. Twenty sheeps with body weigth 29.66±2.74 kg and variance coefficient <10% were used in this research using Completely Randomize Design (CRD). The treatments were consists of four level sweet orange addition in ration. The treatments were T1 (0%), T2 (7%), T3 (12%) and T4 (17%) that were repeated 5 times. The research was conducted for five weeks with observed variables are ration consumption that was measured everyday, body gain that was measured every week, cholesterol and triglycerides measured at the end of the research. During the research water consumption was given ad libitum. Meat samples were selected randomly from three for each repeated treatments. Observation result showed the highest decrease on meat cholesterol level was T2 treatment with 9.43±0.04 mg/mg on thigh and 9.71±0.04 mg/mg on breast. Meanwhile for fat, the highest decrease was from T4 treatment with 9.70±3.98 ug/mg dan 10.48±1.85 ug/mg. Rumen's pH did not show significant difference (P>0.05) on all treatments with value range between 6.80±0.01 - 6.5±0.02. In conclusion, sweet orange (*Citrus sinensis*) waste is capable to lowering cholesterol level and fat in meat of Padjadjaran sheep.

**Key Words**: Sweet Orange Waste, Meat Cholesterol, Meat Fat, Padjadjaran Sheep

## INTRODUCTION

Animal product usually contains high lipid; therefore nowadays people are more selective to choose their diet. This condition surely affects people preference on animal product. Now, is the right time for an innovation to produce high quality meats that have low content of cholesterol and fat. Herbal materials use as a mean to decreasing cholesterol and fat on blood and meat, have been published so many times, one of the herbal material is sweet orange (*Citrus sinensis*) waste. It has so much potential to decrease cholesterol and fat level on animal meat (Adriani et al. 2014)

One option of fitofarmaka is sweet orange (*Citrus sinensis*) waste , because Indonesia produces 2,355,550 tons *Citrus sinensis* each year, (Ministry of Agriculture 2010), while only about 35-40 % which can be used by consumers, for food and beverage processing industry, whereas the remaining 65% is wasted that can not be utilized. This is a good opportunity in livestock feed

because it has a superior active compound (Mirzae & Naser 2008).

Efforts to reduce cholesterol and triglycerides using fitofarmaka for example adding bay leaf flour (*Syzygium polyanthum* Wight) have done by group of Animal Husbandry student, Universitas Padjadjaran. The result showed that blood cholesterol levels decreased from 297.75±51.73 to 139±12.03 mg/dl, with use 4% bay leaf in ration of Broiler. Other Research using *Citrus sinensis* waste in quail, showed a good result and adding up to 6% can reduce the cholesterol content of the meat from 60.11 to 29.82 mg/dL, (Fadilah 2011) and the optimum dose for decreasing fat and cholesterol in poultry not more than 6 % in ration.

The content of the active compound in citrus waste including volatile oil 0.91%, tannins 0.95%, flavonoids 0.46%, and 0.84% saponin (LKO Unpad 2013).

The active compound in sweet oranges such as tannins, saponins, flavonoids and essential oil is able to inhibit the absorption of cholesterol in the intestine. The presence of tannins will stick or lining the intestine membrane thus inhibiting the absorption of cholesterol (Oluremi et al. 2007).

Essential oils are believed have the function of lowering the activity of Glycerol-3-Phosphate Dehydrogenase (GPHD), an enzyme that plays role in triglycerides synthesis. Essensial oil could inhibit triglyceride synthesis in the liver and small intestine may result to decrease the triglycerides (He et al. 2009). The dominant component compound of *Citrus sinensis* is hesperidin and narirutin classified as flavonoids that act as antioxidant (Peterson et al. 2006)

Hesperidin worked through the mechanism of inhibition the activities of HMG-CoA reductase, so the enzyme activity involved in the biosynthesis of cholesterol is inhibited.

Padjadjaran sheep, is a local breed that has related with Garut sheep from Wanaraja area still in purification for meat products, has white feathers and width ear characteristics (Bandiati et al. 2012). They have high potential as meat source and are highly adaptive.

Attempt to decrease cholesterol and fat levels on Padjadjaran sheep's blood by using waste citrus sinensis was feared to harm the sheep's biological function especially rumen's ecology and the value of hematology would improved.There is a trend to modify the animal cholesterol and fat content in order to produce high quality products. *Citrus sinensis* waste has been reported to reduce blood cholesterol and triglyceride level, therefore this study was conducted with the objectives to study the effect of dietary inclusion of *Citrus sinensis* waste on meat cholesterol and fat of Padjadjaran sheep.

## MATERIALS AND METHODS

### Animal Experimental

The sheep were procured from Animal Breeding Station, Animal Husbandry Faculty Universitas Padjadjaran. The research trial was conducted at Faculty of Animal Husbandry, Universitas Padjadjaran, Indonesia from April 2015 to August 2015. Twenty sheep were used for the study. The sheep were reared in cages having age group between 24-30 months with average body weight 29.66±2.74 kg and coefficient of variation <10 %. The experimental period lasted after five (5) weeks. The concentrate ration was used andgrass was used asfed. The consentrate are rice bran, cassava flour, tofu waste, coconut cake, sweet orange (*Citrus sinensis*) waste, molasses, pollard and cassava peal.The sheep were randomly allocated to four treatment groups as T1, T2, T3 and T4, in a Completely Randomized Design (CRD). The sheep in the first group (T1) were givenrasion without the addition of *Citrus sinensis* while in other groups *Citrus sinensis* was supplemented at the rate of 7, 12, and 17% in T2, T3, T4 respectively.

### Proceesing of *Citrus sinensis*

The *Citrus sinensis* was obtained from the sweet orange processing units located around Bandung, was sun dried (<50ºC) until the moisture content reached 10% and followed with milled with hammer mill at Faculty of Animal Husbandry, Universitas Padjadjaran.

### Samples Collection

Meat samples were collected at the end of experimental period, randomly selected from three sheeps from each treatment group, so the total is 12 healthy individuals (60% of total sheep) was slaughtered with a cutting technique streak (Butterfield 1988).

Halal slaughter method is done after fasting for 22 hours to reduce the slaughter weight variation. Meat samples analyzed were taken from the right side of carcass longisimus dorsi – (LD) muscle and the biceps femoris (BF) muscle, were taken at the thigh. The meat was taken randomly as much as 10 grams each, at multiple locations on longisimus dorsi and biceps femoris.

Purbowati & Suryanto (2000) research showed that LD muscle meat fat content (3.10%) higher than the BF (2.16%) because the thigh muscles more used for moving. There is no significantly different (P>0.05) because the sheep were kept in cages so the motion

**Table 1**. Ration composition, nutrient content and energy

| Feed Ingredients | Nutrient Content(100% Dry matter) | | | |
|---|---|---|---|---|
| | R0 | R1 | R2 | R3 |
| | ------------------------- % ------------------------- | | | |
| Rice Bran | 27.92 | 27.59 | 27.82 | 28.09 |
| Cassava flour | 5.36 | 3.22 | 5.4 | 5.43 |
| Tofu Waste | 0.99 | 1.22 | 1.34 | 1.24 |
| Cococnut Meal | 13.00 | 10.14 | 8.1 | 6.59 |
| LJM (*Citrus sinensis)* | 0.00 | 7.00 | 12.00 | 17.00 |
| Molasses | 3.10 | 3.11 | 3.13 | 3.14 |
| Pollard | 21.65 | 22.76 | 23.25 | 23.65 |
| Cassava peal | 27.98 | 25.32 | 19.53 | 15.58 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 |
| Nutrient Composition | | | | |
| Dry Matter | 85.15 | 85.419 | 85.848 | 86.305 |
| Ash | 7.56 | 7.721 | 7.937 | 8.134 |
| Crude Protein | 12.00 | 12.00 | 12.00 | 12.00 |
| Crude Fat | 5.004 | 4.929 | 4.903 | 4.903 |
| Crude Fiber | 10.955 | 11.16 | 11.205 | 11.312 |
| Nitrogen Free Extract | 51.753 | 51.692 | 52.309 | 52.435 |
| Total Digestible Nutrient | 73.00 | 73.00 | 73.00 | 73.00 |

Source: Caltucated aplication from Winfeed

activities in the thigh muscle is limited, as a result fat tends to stockpiled (Lambuth et al. 1970).

Meat cholestrol was estimated using Cholesterol kit (Biolab), using CHOD-PAP (Cholesterol Oxidase Phenylperoxidase Amino Phenozonphenol) method (Richmond 1973 in Kasturi & Singhania 2014). Meat fat was estimated using Soxhlet method (AOAC 2006).

Estimation of rumen pH (ruminal fluid from slaughtered sheep) was collected and pH was measured using pH-meter.

## Statistical analysis

Data collected were subjected to analysis of variance (ANOVA) (Gaspersz 2006) was used to test the significance of difference between means considered significant at P<0.05.

## RESULT AND DISCUSSION

It can be seen from Table 2, that the feed intake of all treatments are relatively equal, concentrate between 375.19±27.89 to 390.32±8.72 and grass consumption 2438.86±154.74 to 2538.21±210.65. Average daily gain ranged from 8.73-64.28 g. The feed intake and body weigh for all treatments was not significantly different (P>0.05).

Forage dry matter intake can be seen in Table 2. Consumption of dry matter forage and concentrates are relatively the same in all treatment, 400-440 g/head/day and exhausted. Average consumption of feed dry matter is 3.3% of body weight that is in the normal range.

The highest daily weight gain achieved by treatment T3 with body weight gain 64.28 g for 5 weeks (Table 2), with adaptations for 1 week. This illustrates that the provision of *Citrus sinensis* up to 12% shows the highest body weight gain compared to other treatments, is closely related to the active compounds including flavonoids and essential oils to improve metebolisme, and its impact on body weight gain (Hernawan & Adriani 2014).

Triglycerides were decreased in all treatment, its serves an energy reserve used (Malinow et al. 1987; Oluremi et al. 2007), so the blood levels to be reduced. The presence of flavonoids also believed to play a role in suppressing the concentration of triglycerides. Flavonoids are thought to activate cAMP synthesis resulting in increased protein kinase resulting in increased triglyceride hydrolysis so the triglycerides in the blood and liver will reduce (Olivera et al. 2007;

**Table 2**. Feed consumption and body weight

| Parameter | T1 | T2 | T3 | T4 |
|---|---|---|---|---|
| Concentrate consumption (g) | 375.19±27.89[a] | 389.95±18.82[a] | 390.32±8.72[a] | 388.04±15.72[a] |
| Grass consumption (g) | 2438.86±154.74[a] | 2524.64±234.94[a] | 2538.21±210.65[a] | 2560.62±236.15[a] |
| Body Weight (Kg) | 28.34±3.46[a] | 30.72±2.96[a] | 30.37±3.03[a] | 33.35±1.31[a] |

**Table 3**. Blood Cholestrol and Triglyceride Contain

| Parameter | R0 | R1 | R2 | R3 |
|---|---|---|---|---|
| Triglyceride (mg/dl) | 30.0±11.0 | 30.4±7.6 | 19.0±2.0 | 20.5±7.5 |
| Cholesterol (mg/dl) | 126.61±10.96 | 129.65±16.44 | 106.52±12.17 | 120.98±15.98 |

Rusell 2009). Protein kinase activates triglyceride lipase through forforilasi here in after described triglycerides into free fatty acids and glycerol by triglyceride lipase (Rusell 2009).

Cholesterol comes from food into the digestive tract and in the small intestine to be absorbed by enterocytes of the small intestine mucosa, the next will be esterification into cholesterol esters. After that, lipoprotein cholesterol esters will formed chylomicrons, then get into the flow of lymph and end up in the bloodstream (Shepherd 2001; Linder 2006).

The active compounds contained in sweet oranges such as tannins, saponins, flavonoids and essential oil is able to inhibit the absorption of cholesterol in the intestine (Malinow et al. 1987; Francis et al. 2002; Oluremi et al. 2007). The presence of tannin can stick or coat the intestinal membrane thereby inhibiting the absorption of cholesterol (Oluremi et al. 2007). In addition, the saponin in the gastrointestinal tract to form a bond with cholesterol complexes that are difficult absorbed by the intestine so that most cholesterol will come out with feces (Malinow et al. 1987). The presence of flavonoids are also believed to inhibit the absorption of cholesterol by inhibiting the formation of micelles ration so that the absorption of cholesterol settles and can be pressed (Olivera et al. 2007; Gropper et al. 2009).

Table 4 is the major research,the results revealed that dietary inclusion sweet orange waste in all levels decreasing the meat cholesterol and fat content of sheep meat, when compared to the control group (T1). Highest reduction of thigh and breast meat cholesterol level 9.43±0.04 and 9.71±0.04 μg/mg respectively, was significantly different ($P<0.05$) recorded in all treatments, fed 7% citrus waste (T2) in the diet when compared to control groups. Further, the highest decrease in fat content of thigh and breast were 9.70±3.98 and 10.48±1.85 μg/mg respectively, was observed in the group fed 17% citrus waste (T4) in the diet compared to other treatments including control.

Flavonoids also produce multi-enzyme systems such as cytochrome P-450; affecting on lipid metabolism and bile acids. The enzyme cytochrome P-450 has the ability to mediate the formation of bile acids so the amount of bile acids was decreased.

Bile acid decreasing is the main route to eliminate the cholesterol. The presence of flavonoids is also believed to play a role in suppressing the concentration of triglycerides (Table 2). Flavonoids which can activated cAMP synthesis, will increase the kinase protein and also increased the triglyceride hydrolysis, which decreased the blood triglycerides (Olivera et al. 2007).

The decreased lipid profile related with Galleano et al. (2012) study, that flavonoids may have beneficial role in regulating the fatty oxidation and improve adipocyte function, also flavonoids act as antioxidants, thus decreased the cholesterol levels in the blood (Oluremi et al. 2007), by release one hydrogen atom from the group and reducing the formation of free radicals, resulting in the synthesis of 3-hydroxy-3-metilglutaril-CoA (HMG CoA) which serves as a precursor in obstruction of cholesterol formation (Reynertson 2007). Naringenin flavonoid in citrus has been reported prevented the accumulation of adipose, adipocyte hypertrophy and dyslipidemia (Mulvihill et al. 2010).

The cholesterol from food, was entered into the digestive tract and small intestine then absorbed by enterocytes of the small intestine mucosa and will esterified into cholesterol esters. After that, with phospholipids and apolipoprotein, lipoprotein cholesterol esters will forming chylomicrons then get into the flow of lymph and the ending in the bloodstream (Linder 2006).

In addition, the saponin in the gastrointestinal tract forming a complex bond with difficult cholesterol absorbed blood cholesterol, but has no significant difference to blood cholesterol and triglycerides, even there are tendency to decrease from 69.6 mg/dL to 64.00 mg/dL (Adriani et al. 2014) by the intestine so most of cholesterol was release with feces). The presence of flavonoids are also believed to inhibit the absorption of cholesterol by inhibiting the formation of micelles

**Table 4.** Effect of *Citrus sinensis* on meat cholesterol, meat fat and rumen pH

| Parameter | T1 (0%) | T2 (7%) | T3 (12%) | T4 (17%) |
|---|---|---|---|---|
| **Meat Cholesterol** | | | | |
| Thigh (µg/mg) | 11.71±0.03[b] | 9.43±0.04[a] | 10.86±0.15[ab] | 10.43±0.20[ab] |
| Breast (µg/mg) | 11.14±0.04[b] | 9.71±0.04[a] | 10.43±0.17[a] | 10.43±0.21[a] |
| **Meat Fat** | | | | |
| Thigh (µg/mg) | 25.68±1.48[b] | 13.85±1.38[a] | 14.51±1.18[a] | 9.70±3.98[a] |
| Breast (µg/mg) | 24.13±0.41[c] | 16.76±1.46[b] | 16.32±0.98[b] | 10.48±1.85[a] |
| **pH** | | | | |
| Rumen | 6.80±0.01 | 6.82±0.03 | 6.78±0.04 | 6.50±0.02 |

Means within the row with different superscripts are significantly different (P<0.05)

ration so the absorption of cholesterol tend to settle and can be decreased (Olivera et al. 2007; Gropper et al. 2009).

Flavonoids and essential oil is thought to have a role to inhibit the synthesis of cholesterol in the liver. Hesperidin which is the dominant compound in the citrus flavonoid work through the mechanism to inhibite the activity of the HMG-CoA reductaseenzyme involved in cholesterol biosynthesis. Essential oils are also believed have a role inhibiting the reductase enzyme HMG-CoA.The essential oils in the skin of the fruit of Citrus plants genus are limonene, citronellal, geraniol, linalol, α-pinene, mirsen, β-pinene, sabinen, geranil acetate, nonanal, geranial, β-kariofilen, and α-terpineol (Chutia et al. 2009).

In this study, the saponin levels in each treatment using *Citrus sinensis* waste are 0.059, 0.109, and 0.143% respectively; tannin levels 0.067, 0.114, and 0.162% respectively, and the levels of flavonoids 0.032, 0.055, and 0.078% respectively (calculated in 7, 12, and 17%). The levels of these compounds is still tolerate, such as tannin only used not more than 2-3%, and saponins can be used up to 0.2% (Oluremi et al. 2007).

Another study, about the addition of sweet orange waste more than 20% in the diet, has increased the level of saponin, so the ration becomes less palatable, even the rations can not be consumed (Callaway et al. 2010). Tannins will coated the intestine membrane and inhibits the absorption of nutrients include the cholesterol

The essential oils have also been reported to decrease the blood triglyceride levels by decreasing the activity of Glycerol-3- Phosphate dehydrogenase (GPDH) enzyme, which is involved in the biosynthesis of triglycerides, according He et al. 2009. Fat tissue is made up of fat cells in a matrix of connective tissue. Triglycerides content in fat cells make up around 85% of the fat tissue, and triglyceride is made up of three fatty acids; can be any of many combinations of saturated, mono-unsaturated and polyunsaturated acids.

Most of ruminant, lipids consumed will hydrolysed in the intestine with helped bile salts and pancreatic lipase to fatty acids and glycerol. Lipids are already partially digested mainly in the form water-soluble, forming micelle is stable, especially content long chain fatty acids, monoglycerides and bile acids are diffused into the surface of mucosal cells and release the material to be absorbed (Linder 2006).

Additionally, apart from the effect on meat cholesterol and fat, the effect of citrus waste on rumen pH was also studied. The results revealed that the values ranged from 6.80±0.01 to 6.50±0.02 among various treatment groups including control group (Table 4). There was no statistical difference in rumen pH among all treatments, indicating that adding of citrus waste up to 17% level had no detrimental effect on rumen ecology.

Results of this study are very important because it can decrease the cholesterol meat level until 19.47% in thigh meat compared to the control using 7%, and also decreased the meat fat 62.22% when using 17% *Citrus sinensis* waste.

## CONCLUSION

It could be concluded that the dietary added with *Citrus sinensis* waste in sheep had beneficial effects with regard to its ability in reducing the meat cholesterol and fat levels which could be attributed to various active ingredients present in it. Further, the inclusion of *Citrus sinensis* waste up to 17% level in the diet of sheep had no detrimental effect on rumen ecology in terms of rumen pH levels. However, further studies in this regard with different inclusion levels are warranted.

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# Voice Characteristics of Some Sheep: Utilization to Estimation of Genetic Distance

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**ABSTRAK**

Handiwirawan E, Noor RR, Sumantri C, Subandriyo. 2015. Karakteristik suara beberapa rumpun domba dan pemanfaatannya untuk pendugaan jarak genetik. JITV 20(4): 257-267. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1274

Analisa suara telah dilakukan dalam berbagai kegunaan diantaranya identifikasi dan pembedaan spesies serta penyusunan taksonomi pada beberapa spesies hewan karena beberapa kelebihan, diantaranya tidak perlu menangkap atau terlalu dekat dengan subjek yang diamati. Analisa suara yang digunakan untuk pembedaan dan pendugaan jarak genetik rumpun domba belum pernah dilaporkan. Penelitian ini dilakukan untuk mempelajari karakter suara beberapa rumpun domba dan kemungkinannya dipergunakan sebagai alat penduga jarak genetik antar rumpun domba. Penelitian dilakukan di Kandang Percobaan Domba, Balai Penelitian Ternak, Bogor. Sebanyak 20 ekor dari lima rumpun domba dewasa (St. Croix cross/SC, Barbados Black Belly cross/BC, Lokal Garut/LG, Komposit Garut/KG dan Komposit Sumatera/KS) digunakan dalam penelitian ini. Suara panggilan (*call sound*) direkam menggunakan alat digital *voice recorder*. Analisa suara dilakukan dengan Software Raven Pro 1.3 for Windows untuk menghitung sebanyak 24 peubah suara. Analisa ragam dari setiap peubah suara dilakukan menggunakan PROC GLM dari software SAS Ver. 9,0. PROC CANDISC digunakan untuk analisa diskriminan kanonikal dan selanjutnya PROC TREE digunakan untuk membangun dendogram. Hasil analisa suara menunjukkan bahwa di antara kelima rumpun domba terdapat variasi dalam peubah amplitudo, energi, daya dan frekuensi. Berdasarkan plotting kanonikal, domba LG, KS dan BC merupakan rumpun domba yang berbeda kelompok. Dari hasil penelitian disimpulkan bahwa peubah-peubah karakteristik suara yang dapat digunakan sebagai pembeda rumpun domba adalah frekuensi kuartil ketiga, frekuensi tengah, frekuensi maksimum dan waktu frekuensi kuartil pertama. Dendogram yang dibangun menempatkan rumpun domba KG pada kelompok yang kurang akurat. Metode pendugaan jarak genetik dengan menggunakan data karakteristik suara mempunyai peluang untuk diaplikasikan.

**Kata Kunci**: Karakteristik, Suara Panggilan, Pembedaan, Jarak Genetik, Domba

**ABSTRACT**

Handiwirawan E, Noor RR, Sumantri C, Subandriyo. 2015. Voice characteristics of some sheep: Utilization to estimation of genetic distance. JITV 20(4): 257-267. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1274

Sound analysis has been carried out in various activities including identification and differentiation of species as well as the preparation of the taxonomy of some animals' species because of several advantages, including no need to capture or too close to the subject observed. Analysis of voice used to differentiate and to estimate of breeds' sheep genetic distance has not been reported. This research was conducted to study the character of a few breeds' sheep sound and likely to be used as a predictor of genetic distance between breeds of sheep. The study was conducted in the Animal House at Indonesian Research Institute for Animal Production, Bogor. A total of 20 head adult of five sheeps (St. Croix cross/SC, Barbados Black Belly cross/BC, Local Garut/LG, Composite Garut/KG and Composites Sumatra/KS) used in this study. Call sound recorded using a digital voice recorder. Sound analysis performed by Raven Software Pro 1.3 for Windows to count as many as 24 variables sound. Analysis of variance of each variable sound was performed using PROC GLM of SAS software Ver. 9.0. It used PROC CANDISC for canonical discriminant analysis and then PROC TREE to build a dendogram. The results showed that there were variations in amplitude, energy, power and frequency variables among the five breeds of sheep. By plotting canonical, LG, KS and BC sheep were from a different group. It was concluded that the sound characteristics variables which can be used as a differentiator breeds of sheep were the third quartile frequency, center frequency, maximum frequency and the first quartile time. Dendogram showed that KG sheep was in the less accurate group. Genetic distance estimation method using voice characteristic data may be applied on sheep.

**Key Words**: Characteristics, Call Voice, Differentiation, Genetic Distance, Sheep

## INTRODUCTION

In the context of animal behavior, communication is the sharing of information between two or more individual animals (Scott 2005). All animals communicate with a combination of visual, auditory and olfactory/chemical transmission and through physical contact. Communication is critical in the survival of individuals and species because it has a relationship with the protection (Suzuki 2014), reproduction, and an introduction of mother-to-child (Sèbe et al. 2010). Voice is one important way of communicating among many animal species.

Vocalization in mammals consists of a number of different call types (Fitch et al. 2002). In a voice animal contains some of information about the animal, including an identity (Price et al. 2009), social ranking (Vannoni & McElligott 2008), age, sex and size (Hall et al. 2013; Ey et al. 2007).

It has also been understood that the behavior of a cow may be used as an indicator of mental and physiological conditions (Manteuffell et al. 2004) until the level of stress to assess the status of animal welfare. Engeldal et al. (2012) have reported that social isolation on several breeds of sheep led to changes in the characteristics of the resulting vocalization, as well as in goats (Siebert et al. 2011). Voice analysis have potential as a tool monitors the welfare of cows (Meen et al. 2015), as well as observation of voice activity in the water on the whale that "caged" was an effective method to monitor the level of stress (Castellote & Fossa 2006).

Some previous researchers have reported the use of voice analysis in a variety of purpose for identification, differentiation of species and taxonomic. Ruppell (2010) have studied the diversity of voices two Gibbon population in Vietnam and Laos and assess the taxonomic relationships among both populations. The results of sound analysis conducted Gogala & Trilar (2004) has proposed changes to the taxonomy of crickets under consideration behavior vocalization. In birds, chirping voice difference was most reliable criterion in the differentiation of species of birds (Mahler & Gil 2009).

Voice analysis has been utilized in supporting the genetic and morphological data to reconstruct the evolutionary history of species of Woodpecker birds (Benz & Robbins 2011). Identification and differentiation of bird species are separated geographically by songbird have been successfully conducted and reported by Ohya (2004) in bird of Tibicen in Japan, Mena & Mora (2011) in bird of Cuban Toby (*Todus multicolor*) in Cuba, Lovell & Lein (2013) in bird of Alder flycatchers (*Empidonax alnorum*). Squirrels difficult to distinguish by its morphology, Esser et al. (2008) have reported that the voice analysis successfully used to identify and distinguish the species of squirrel. Ranft (2004) suggested that between the uses of voice analysis is for the description, comparison and analysis of voice; identification of species, populations and individuals; taxonomy and systematics; luring and trapping, and prevention of pest.

The use of voice analysis in various scientific activities has several advantages, including one non-invasive method that does not need to catch or get too close to the subject observed. With the current recording equipment, data recording allows to be obtained in which the subject is not visible or invisible but it is not clear, for example, because the subject is hidden in the forest or on observations done at night for nocturnal animals (Burton & Nietsch 2010). To facilitate the work, now the identification of the species is possible to do automatically (Chesmore 2004).

Analysis of voice used for the differentiation and genetic distance estimation breeds of sheep has not been reported. Voice change due to hybridization to understand the processes that lead to speciation species have been studied by Dere´gnaucourt (2010) in Quail. Studies conducted Rheindt et al. (2008) showed that based on DNA evidence, vocalizations may be a better indicator for taxonomy than a feather pattern. Based on previous research on these birds, the voice characterization studies conducted in several breeds of sheep that are genetically have a relationship to predict of genetic distance.

This research was conducted to study the voice character of a few breeds of sheep and likely used as a tool the genetic distance between breeds of sheep. The results are expected to be used as an alternative in the estimation of genetic distances in sheep.

## MATERIAL AND METHOD

The study was conducted in two Cage Experiment Sheep in Cilebut and Bogor at Indonesian Research Institute for Animal Production. The equipment used was a digital voice recorder which records the sound of sheep in the MP3 file format. Sheep used in the research was the adult sheep (aged 2-9 years) from five breeds of sheep namely St. Croix cross (SC, 50% Local Sumatra 50% St. Croix), Barbados Black Belly cross (BC, 50% Local Sumatra 50% Barbados Black Belly), Local Garut (LG), Composite Garut (KG, 50% Local Garut 25% St. Croix 25% Moulton Charolais) and Composite Sumatra (KS, 50% Local Sumatra 25% St. Croix 25% Barbados Black Belly). The amount of each breeds of sheep used in this study were 20 heads (5 males and 15 females in status after weaning).

Each of the sampled sheep separated from sheep group to another empty cage to stimulate the sheep give a call voice. The duration of observation to each sheep

varied until collected about 5-10 record voice calls. Before further analysis, the voice sample was cleaned from noise, hiss and the voices that are not desired (such as footsteps, the sound of other sheep, etc.), using Wavepad Sound Editor software Ver. 4.28. Sheep voice samples were mixed with the other sheep was not used in the analysis. The average of three voice recording from each head then analyzed further. Sheep voice analysis done with Sound Analysis Software Raven Pro 1.3 for Windows; software created by the Cornell Laboratory of Ornithology; downloaded and purchased from the website http://birds.cornell.edu/. The voice of sheep from the five breeds was translated by Raven 1.3 Pro software in the form of spectrogram and waveform.

Voice analysis by Raven Software 1.3 Pro (Charif et al. 2008), describes the voice characteristics of the each of five breeds. Variables were measured:

1. Delta Time (DELTIME) = Difference between begin time and end time for the selection (Units: seconds).
2. Length of Waveform (LWAVE) = Number of frames contained in a selection. For waveform views, the number of frames equals the number of samples in a single channel (Units: frames).
3. Maximum Amplitude (MAXAMP) = Maximum of all the sample values in the selection (Units: dimensionless sample values).
4. Maximum Amplitude Time (MAXAMPT) = First time in the selection at which a sample with amplitude equal to max amplitude occurs (Units: seconds).
5. Minimum Amplitude (MINAMP) = Minimum of all sample values in the selection (Units: dimensionless sample values).
6. Minimum Amplitude Time (MINAMPT) = First time in the selection at which a sample with amplitude equal to min amplitude occurs (units: seconds)
7. Peak Amplitude (PAMP) = Greater of the absolute values of max amplitude and min amplitude (Units: dimensionless).
8. Peak Amplitude Time (PAMPT) = First time in the selection at which a sample with amplitude equal to Peak Amplitude occurs (Units: seconds).
9. RMS Amplitude (RMSAMP) = Root-mean-square amplitude of the selected part of the signal (Units: dimensionless sample units).
10. 1st Quartile Frequency (Q1FREQ) = Frequency that divides the selection into two frequency intervals containing 25% and 75% of the energy in the selection (Units: Hz).
11. 1st Quartile Time (Q1TIME) = Point in time that divides the selection into two time intervals containing 25% and 75% of the energy in the selection (Units: seconds).
12. 3rd Quartile Frequency (Q3FREQ) = Frequency that divides the selection into two frequency intervals containing 75% and 25% of the energy in the selection (Units: Hz).
13. 3rd Quartile Time (Q3TIME) = Point in time that divides the selection into two time intervals containing 75% and 25% of the energy in the selection (Units: seconds).
14. Average Power (AVGPOW) = Value of the power spectrum averaged over the frequency extends of the selection (Units: dB).
15. Center Frequency (CENTFREQ) = Frequency that divides the selection into two frequency intervals of equal energy (Units: Hz).
16. Center Time (CENTTIME) = Point in time at which the selection is divided into two time intervals of equal energy (Units: seconds).
17. Energy (ENERGY) = The total energy within the selection bounds (Units: dB).
18. IQR (Inter-quartile range) Bandwidth (IQRBW) = Difference between the 1st and 3rd Quartile Frequencies (Units: Hz).
19. IQR (Inter-quartile Range) Duration (IQRDUR) = Difference between the 1st and 3rd Quartile Times (Units: seconds).
20. Length of Spectrogram (LSPECT) = The number of frames contained in a selection. For spectrogram and spectrogram slice views, the number of frames equals the number of individual spectra in the selection in one channel (Units : frames).
21. Maximum Frequency (MAXFREQ) = Frequency at which max power occurs within the selection (Units: Hz).
22. Maximum Power/Peak Power (MAXPOW) = Maximum power in the selection (Units: dB).
23. Maximum Power Time (MAXPOWT) = First time in the selection at which a sample with power equal to Max Power occurs (Units: seconds).
24. Maximum Frequency Time (MAXFREQT) = First time in the selection at which a sample with power equal to Max Frequency occurs (Units: seconds).

### Data Analysis

Prior statistical analyzes were performed, each value of the variable to be corrected for ewe. PROC GLM of SAS software Ver. 9.0 was used to obtain the value of the correction factor for sex. The least square means (LSM) on the results of analysis of variance was used to determine a correction factor. The correction factor for the sexes was calculated by adding or subtracting LSM of data.

Normal distribution test was conducted by the Kolmogorov-Smirnov test. Analysis of variance of each variable voice was performed using SAS software Ver. 9.0 with PROC GLM, and performed significance test

to see the difference between the breeds of sheep. Model of linear equations used were:

$$Y_{ij} = \mu + B_i + \varepsilon_{ij}$$

which is:
$Y_{ij}$ = The observation of the i-th breed
j-th = Replication
$\mu$ = The population mean
$B_i$ = The effect of the i-th breed (i = 1, 2, 3, 4, 5)
$\varepsilon_{ij}$ = A random error due to the effect of the i-th breed and the j-th replication

PROC CANDISC of SAS software ver 9.0 used to perform the canonical discriminant analysis to calculate the Mahalanobis distance, canonical coefficients and provides a visual interpretation of differences in breeds of sheep. Based on Mahalanobis distance matrices that have resulted from previous analysis, PROC CLUSTER with Average Linkage method (Unweight Pair-Group Method Using Arithmetic Averages, UPGMA) perform hierarchical clustering. From the resulting output was then made dendogram to five breeds of sheep with PROC TREE of SAS software ver 9.0 (SAS 2002).

## RESULT AND DISCUSSION

### Variation Voice Characteristics

The 24 variables voice characteristics of breeds of sheep that can be calculated using voice analysis software Raven Pro 1.3 was shown in Table 1. It can be seen that duration of sheep voice varied between 0.96 to 1.52 seconds. Duration of sheep voice of SC and KG were no different, but the both sheep were different with sheep BC, LG and KS.

SC sheep had a voice with maximum amplitude (29584.8 units) while the lowest was KS sheep (24162.3 units). Voice energy of LG sheep (102.3 dB), SC sheep (101.4 dB) and KG (99.9 dB) were not significant different but higher than BC sheep (97.1 dB) that equal to KS sheep (97.7 dB). Power Maximum of voice of BC and KS sheep was lower than SC and LG sheep. Variable of amplitude, energy and power related to the strength or weakness of the sound produced from each breeds of sheep. Engeldal et al. (2012) have reported that the difference in amplitude, energy and power of the sound produced, in addition to genetically influenced was also influenced by the environment (social isolation). Voice amplitude of Bison was closely related to competitive ability of bull (quality, condition and motivation), and thus, can be a selection signal for male sexual performance. Wyman et al. (2008) on the results of his research on Bison reported that there was a positive relationship between the voice amplitude, good physical condition and motivation, otherwise there was a negative relationship between the voice amplitude and quality of bull (mating and reproductive success).

Frequency variable indicates the high and low tone of voice. The results showed that the maximum frequency of LG sheep (1202.5 Hz) equal to SC sheep (1408.5 Hz), but lower than KS sheep (1800.6 Hz) and BC sheep (1642.7 Hz). Shillito-Waser & Hague (1980) also found and reported that there was a difference some voice parameter of high-pitched sound between Clunt Forest, Jakob, Dalesbred and Border Leicester sheep. Some specific voice in sheep that indistinguishable was low-pitched voice is usually generated by lamb and high-pitched voice usually produced by ewes and also as a protest or miserable / sad voice (Krause & Ruxton 2002). Some researchers reported a negative correlation between voice frequency and body size. The results of the study were reported in the species of bats (Zhang et al. 2000) and birds (Brumm & Naguib 2009; Martin et al. 2011) as well as between species (Fletcher 2010). Allometric relationship between the voice frequency and body size arises because of physical and energy constraints; animals cannot efficiently produce voice waves that are larger than the body size or the apparatus of their voice production (Bradbury & Vehrencamp 1998). This is opens an opportunity of indirect selection on the production traits of body weight by utilizing the voice frequency data.

The ability to produce voice depends on the existence of special elements of trachea; in mammals; pharyngeal cavity. Anatomy and function of the acoustic element determines the range, the acoustic characteristics and limits of voice production within species (Manteuffel et al. 2004). Changes that occur in an animal may be as a behavior reaction and or physiology that can be measured and used to describe the state of individual and the specific meaning of the voice. This makes the sound produced was useful as a tool to assess the status of well-being and stress on the individual animal (Weary & Fraser 1995).

Visualize the sound of five sheep in the two forms of picture shown in Figure 1. The first visualization was in a waveform (top) and the second was a spectrogram (bottom). Waveform shape is visually describing voice in amplitude (vertical axis) versus time (horizontal axis). Meanwhile, visualization in a spectrograms describe voice in a frequency (vertical axis) versus time (horizontal axis) and power relatively at any point frequency and time indicated by the color of white, gray to black. The darker of dot indicate that power was getting stronger, so the darkest point was representing a maximum power in the voice. Visually difference in the five sheeps is shown in Figure 1.

**Table 1**. Least square means of some waveform and spectrogram variables of voice from Barbados Black Belly Cross (BC), Local Garut (LG), Composite Garut (KG), Composite Sumatera (KS) and St. Croix (SC) sheep

| Variables | Breed of Sheep | | | | |
|---|---|---|---|---|---|
| | SC | BC | LG | KG | KS |
| DELTIME (second) | 1.15±0.06$^{b}$ | 0.92±0.06$^{c}$ | 1.52±0.06$^{a}$ | 1.13±0.05$^{b}$ | 0.96±0.05$^{c}$ |
| LWAVE (frame) | 36270.70±1748.84$^{b}$ | 29332.09±1748.84$^{c}$ | 48251.90±1748.84$^{a}$ | 36098.80±1704.56$^{b}$ | 30558.41±1663.48$^{c}$ |
| MAXAMP (unit) | 29584.77± 1335.10$^{a}$ | 25810.82±1335.10$^{bc}$ | 29245.16±1335.10$^{ab}$ | 26904.14±1301.30$^{abc}$ | 24162.28±1269.94$^{c}$ |
| MAXAMPT (second) | 0.36±0.03$^{b}$ | 0.47±0.03$^{a}$ | 0.52±0.03$^{a}$ | 0.48±0.03$^{a}$ | 0.35±0.03$^{b}$ |
| MINAMP (unit) | 28998.03±1339.35$^{b}$ | 24910.87±1339.35$^{a}$ | 29161.82±1339.35$^{b}$ | 27022.66±1305.44$^{ab}$ | 24010.97±1273.97$^{a}$ |
| MINAMPT (second) | 0.39±0.04$^{ab}$ | 0.46±0.04$^{bc}$ | 0.55±0.04$^{cd}$ | 0.47±0.04$^{bcd}$ | 0.36±0.03$^{a}$ |
| PAMP (unit) | 30145.85±1327.002$^{a}$ | 26431.83±1327.002$^{ab}$ | 29742.96±1327.002$^{a}$ | 27719.09±1293.40$^{ab}$ | 24846.21±1262.23$^{b}$ |
| PAMPT (second) | 0.37±0.03$^{c}$ | 0.47±0.03$^{b}$ | 0.56±0.03$^{a}$ | 0.48±0.03$^{b}$ | 0.35±0.03$^{c}$ |
| RMSAMP (unit) | 7206.82±386.86$^{a}$ | 5229.33±386.86$^{b}$ | 7529.58±386.86$^{a}$ | 6077.97±377.07$^{b}$ | 5444.41±367.98$^{b}$ |
| Q1FREQ (Hz) | 986.61±60.67$^{bc}$ | 1169.72±60.67$^{a}$ | 867.83±60.67$^{c}$ | 1099.10±59.14$^{ab}$ | 1249.48±57.71$^{a}$ |
| Q1TIME (second) | 0.33±0.02$^{b}$ | 0.36±0.02$^{b}$ | 0.42±0.02$^{a}$ | 0.35±0.02$^{b}$ | 0.28±0.02$^{c}$ |
| Q3FREQ (Hz) | 1908.07±66.70$^{b}$ | 2441.25±66.70$^{a}$ | 1824.96±66.70$^{ab}$ | 1969.11±65.01$^{b}$ | 2304.66±63.44$^{a}$ |
| Q3TIME (second) | 0.72±0.04$^{bc}$ | 0.63±0.04$^{ab}$ | 0.96±0.04$^{d}$ | 0.73±0.04$^{c}$ | 0.57±0.04$^{a}$ |
| AVGPOW | 79.88±0.86$^{a}$ | 76.56±0.86$^{b}$ | 79.65±0.86$^{a}$ | 78.25±0.84$^{ab}$ | 76.82±0.82$^{b}$ |
| CENTFREQ (Hz) | 1492.37±64.26$^{bc}$ | 1875.38±64.26$^{a}$ | 1360.77±64.26$^{c}$ | 1601.79±62.63$^{b}$ | 1872.44±61.12$^{a}$ |
| CENTTIME (second) | 0.51±0.03$^{b}$ | 0.48±0.03$^{bc}$ | 0.66±0.03$^{a}$ | 0.55±0.03$^{b}$ | 0.41±0.03$^{c}$ |
| ENERGY (dB) | 101.40±0.96$^{a}$ | 97.13±0.96$^{c}$ | 102.26±0.96$^{a}$ | 99.92±0.93$^{ab}$ | 97.75±0.91$^{bc}$ |
| IQRBW (Hz) | 921.48±64.25$^{b}$ | 1271.55±64.25$^{a}$ | 957.12±64.25$^{b}$ | 812.35±62.62$^{b}$ | 995.37±61.11$^{b}$ |
| IQRDUR (second) | 0.41±0.03$^{b}$ | 0.31±0.03$^{c}$ | 0.56±0.03$^{a}$ | 0.41±0.03$^{b}$ | 0.31±0.03$^{c}$ |
| LSPECT (*frame*) | 282.25±13.44$^{b}$ | 229.21±13.44$^{c}$ | 376.93±13.44$^{a}$ | 287.02±13.10$^{b}$ | 239.92±12.79$^{c}$ |
| MAXFREQ (Hz) | 1408.45±82.51$^{ab}$ | 1642.69±82.51$^{cd}$ | 1202.48±82.51$^{a}$ | 1540.55±80.42$^{bc}$ | 1800.62±78.48$^{d}$ |
| MAXPOW (dB) | 101.96±0.82$^{a}$ | 99.25±0.82$^{b}$ | 101.91± 0.82$^{a}$ | 100.96± 0.80$^{ab}$ | 98.78±0.78$^{b}$ |
| MAXPOWT (second) | 0.43±0.04$^{bc}$ | 0.46±0.04$^{bc}$ | 0.57±0.04$^{a}$ | 0.51±0.04$^{ab}$ | 0.39±0.04$^{c}$ |
| MAXFREQT (second) | 0.43±0.04$^{bc}$ | 0.46±0.04$^{bc}$ | 0.58±0.04$^{a}$ | 0.51±0.04$^{ab}$ | 0.38±0.04$^{c}$ |

Different small letters on the same line showed significant differences ($P<0.05$)

DELTIME = Delta Time; LWAVE = Length of Waveform; MAXAMP = Maximum Amplitude; MAXAMPT = Maximum Amplitude Time
MINAMP = Minimum Amplitude; MINAMPT = Minimum Amplitude Time; PAMP = Peak Amplitude; PAMPT = Peak Amplitude Time
RMSAMP = RMS Amplitude; Q1FREQ = 1st Quartile Frequency; Q1TIME = 1st Quartile Time; Q3FREQ = 3rd Quartile Frequency
Q3TIME = 3rd Quartile Time; AVGPOW = Average Power; CENTFREQ = Center Frequency; CENTTIME = Center Time
ENERGY = ENERGY; IQRBW = IQR (Inter-quartile range) Bandwidth; IQRDUR = IQR (Inter-quartile Range) Duration; LSPECT = Length of Spectrogram
MAXFREQ = Maximum Frequency; MAXPOW = Maximum Power/Peak Power; MAXPOWT = Maximum Power Time; MAXFREQT = Maximum Frequency Time

Table 2 shows a correlation between explanatory variables of voice characteristics and canonical discriminant function. The higher of correlation value indicates that the variable is closely associated with the differences in breeds of sheep. Based on the analysis of canonical structure variables for voice characteristics, there are several major variables that a key differentiator for breeds of sheep. Variables as a differentiator for sheep was Q3FREQ, CENTFREQ, and MAXFREQ (canonical 1) with a value of 0.700361, 0.670637 and 0.526933 respectively and Q1TIME (canonical 2) with a value of 0.515125 (Table 2). The differentiator variables are a variables related to the frequency of voice that indicates high or low tone of voice. It shows that the five breeds of sheep were observed can be distinguished based on the high and low tone of the voice produced, as has also been discovered and reported by Shillito-Waser and Hague (1980), his work for Clunt Forest, Jakob, Dalesbred and Border Leicester sheep.

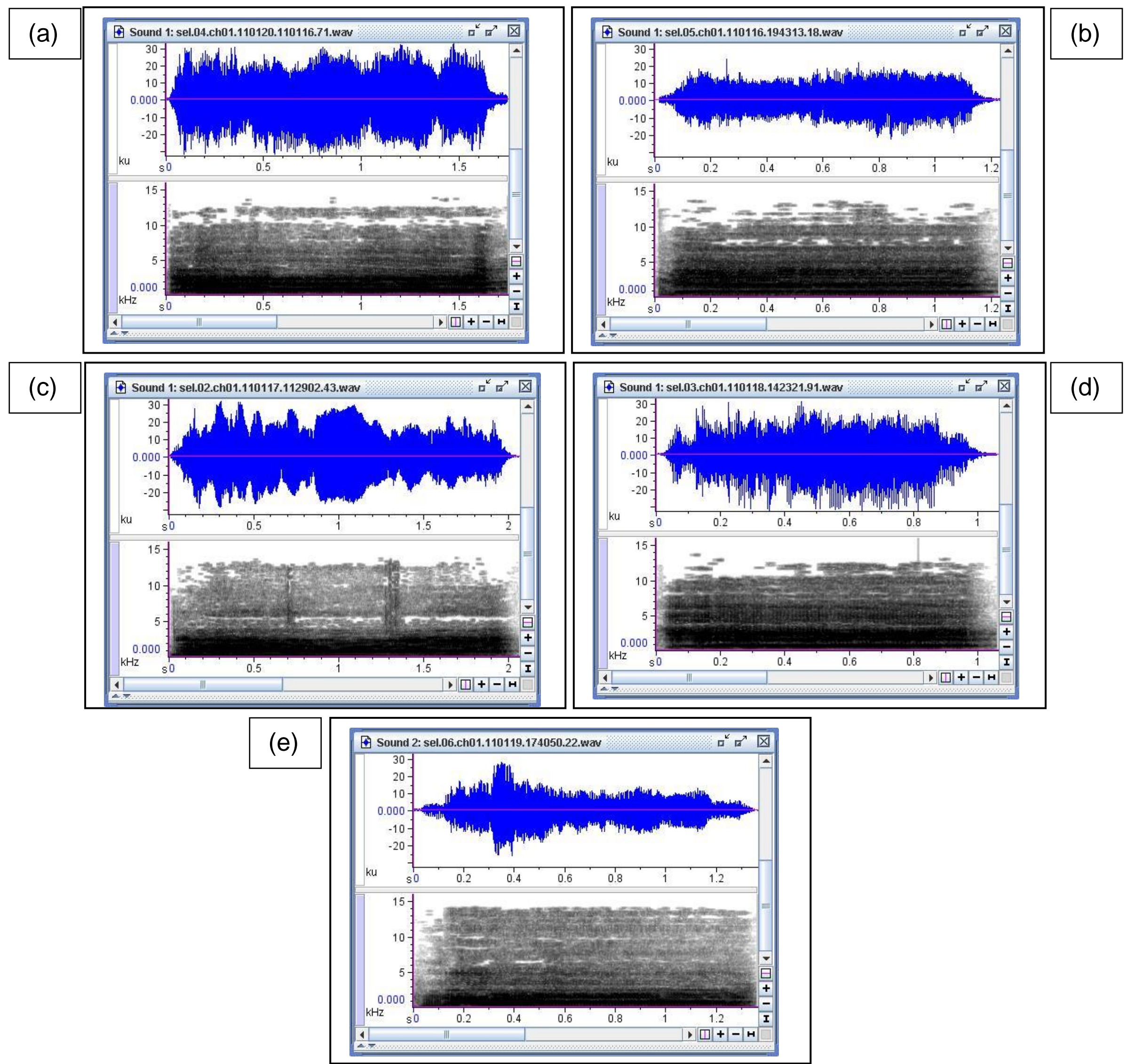


**Figure 1**. Sample of waveform (top) and spectrogram (below) image for St. Croix Cross (a), Barbados Black Belly Cross (b), Local Garut (c), Composite Garut (d) and Composite Sumatra (e) sheep

**Table 2**. Structure of canonical for voice characteristics variables of Barbados Black Belly Cross (BC), Local Garut (LG), Composite Garut (KG), Composite Sumatra (KS) and St. Croix Cross (SC) sheep

| Explanatory variables | Canonical 1 | Canonical 2 | Canonical 3 | Canonical 4 |
|---|---|---|---|---|
| DELTIME (second) | -0.804159 | 0.172014 | 0.020800 | 0.028126 |
| LWAVE (*frame*) | -0.804284 | 0.174587 | 0.008100 | 0.057411 |
| MAXAMP (unit) | -0.296069 | 0.148056 | 0.325895 | -0.098517 |
| MAXAMPT (second) | -0.217527 | 0.381652 | -0.117328 | 0.488773 |
| MINAMP (unit) | 0.337187 | -0.100833 | -0.304504 | 0.036667 |
| MINAMPT (second) | -0.284509 | 0.350645 | -0.097814 | 0.314588 |
| PAMP (unit) | -0.288600 | 0.137820 | 0.329980 | -0.073348 |
| PAMPT (second) | -0.352241 | 0.442150 | -0.170280 | 0.462379 |
| RMSAMP (unit) | -0.531255 | 0.064922 | 0.301289 | -0.213022 |
| Q1FREQ (Hz) | 0.492905 | -0.226207 | -0.235607 | 0.082680 |
| Q1TIME (second) | -0.418608 | 0.515125 | 0.012915 | 0.252081 |
| Q3FREQ (Hz) | 0.700361 | 0.080604 | -0.451466 | -0.128122 |
| Q3TIME (second) | -0.730589 | 0.305336 | 0.052940 | 0.151321 |
| AVGPOW (dB) | -0.351557 | 0.017223 | 0.309296 | -0.107666 |
| CENTFREQ (Hz) | 0.670637 | -0.105598 | -0.375400 | -0.017053 |
| CENTTIME (second) | -0.614365 | 0.350807 | 0.062471 | 0.321176 |
| ENERGY (dB) | -0.490680 | 0.023601 | 0.287804 | -0.050939 |
| IQRBW (Hz) | 0.346765 | 0.402662 | -0.277060 | -0.315448 |
| IQRDUR (second) | -0.744970 | 0.191884 | 0.077126 | 0.100050 |
| LSPECT (*frame*) | -0.810407 | 0.162897 | 0.006552 | 0.106825 |
| MAXFREQ (Hz) | 0.526933 | -0.279258 | -0.242992 | 0.029671 |
| MAXPOW (dB) | -0.342765 | 0.077705 | 0.340077 | 0.013473 |
| MAXPOWT (second) | -0.333054 | 0.248247 | -0.022278 | 0.317889 |
| MAXFREQT (second) | -0.364816 | 0.271570 | -0.025657 | 0.323383 |

DELTIME = Delta Time; LWAVE = Length of Waveform; MAXAMP = Maximum Amplitude
MAXAMPT = Maximum Amplitude Time; MINAMP = Minimum Amplitude; MINAMPT = Minimum Amplitude Time
PAMP = Peak Amplitude; PAMPT = Peak Amplitude Time; RMSAMP = RMS Amplitude
Q1FREQ = 1st Quartile Frequency; Q1TIME = 1st Quartile Time; Q3FREQ = 3rd Quartile Frequency
Q3TIME = 3rd Quartile Time; AVGPOW = Average Power; CENTFREQ = Center Frequency
CENTTIME = Center Time; ENERGY = ENERGY; IQRBW = Inter-Quartile Range Bandwidth
IQRDUR = IQR (Inter-quartile Range) Duration; LSPECT = Length of Spectrogram; MAXFREQ = Maximum Frequency
MAXPOW = Maximum Power/Peak Power; MAXPOWT = Maximum Power Time; MAXFREQT= Maximum Frequency Time
Canonical (canonical discriminant function) = value of correlation between an explanatory variables and discriminant function

**Differentiation of sheep based on voice characteristics**

Plotting canonical shows graphically the grouping breeds of sheep was shown in Figure 2. Based on the canonical plotting voice characteristics, LG, KS and BC sheep were different groups of breed, while SC, KG and KS sheep were the one group sheep (visible coinciding on Figure 2). It means that SC, KG and KS sheep were in one group so has the voice characteristic relatively similar. However, the LG, KS and BC sheep have the different voice characteristics and it relates to the genetic similarity among sheeps. Differences between breeds of sheep in particular were at variable of frequencies, as shown in Table 2.

Mahalanobis distance values among the five sheeps observed were listed in Table 3. Based on the characteristics of voice, the closest genetic distance among of sheep was between SC and KG sheep with a value of 4.06042 and the probability distance was not significant (P>0.05). This means that the genetic distance of the two clusters of sheep are very close so it is not a separate sheep. The breed sheep that has a value closest genetic distance with the two breeds of sheep (KG and SC) were KS sheep, the closeness of three sheeps was also demonstrated by coinciding plotting in Figure 2. The closeness and relationship of three breeds because each breeds of sheep genetically have genes from a St. Croix sheep, meanwhile the farthest genetic

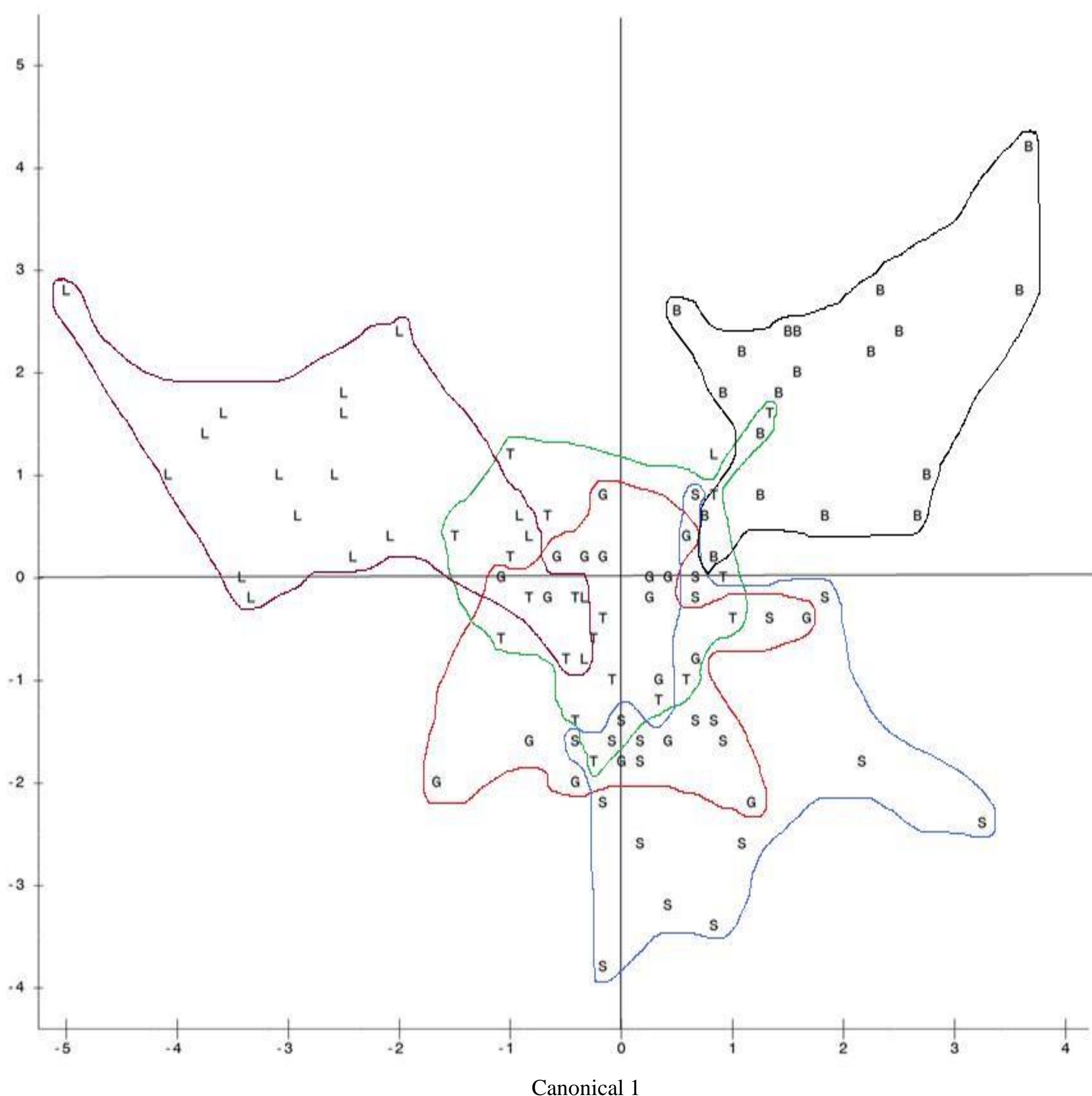


**Figure 2**. Plotting canonical illustrates grouping five breeds of sheep by the voice character (symbol letters B = BC, L = LG, G = KG, S = KS, T = SC)

**Table 3**. Mahalanobis distance value and significance probabilities between five breeds of sheep

| Breed of sheep | BC | KG | LG | KS | SC |
|---|---|---|---|---|---|
| BC | 0 | 11.02691 | 18.27899 | 13.60458 | 11.27703 |
| KG | <.0001 | 0 | 10.07305 | 5.45791 | 4.06042 |
| LG | <.0001 | 0.0001 | 0 | 15.81412 | 10.31253 |
| KS | <.0001 | 0.0364 | <.0001 | 0 | 7.93047 |
| SC | <.0001 | 0.2399 | 0.0001 | 0.0017 | 0 |

Values above the diagonal shows the value of Mahalanobis distance
Values below the diagonal indicate significance probability of Mahalanobis distance

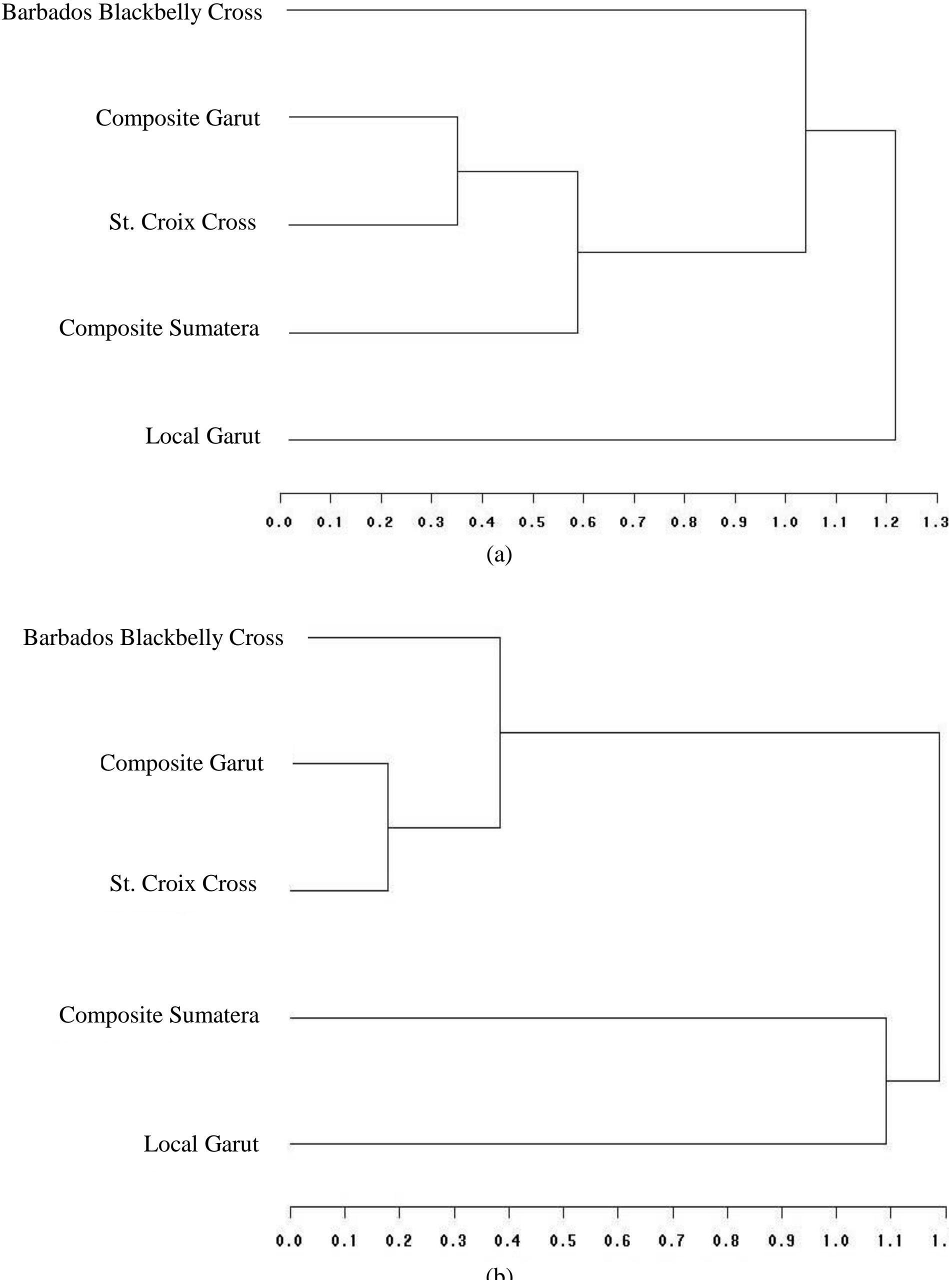


**Figure 3**. Comparison of dendrogram constructed based on the Mahalanobis distance of five sheeps based on data from the voice characteristics (a) and which have been reported by Handiwirawan et al. (2012) based on the body size (b)

distance shown between BC and LG sheep, with a value of 18.27899. This is because the two of sheeps (BC and LG sheep) have no common ancestor so they were not related genetically.

A dendogram (Figure 3) were made to clarify the relationship between the groupings of the five breeds of sheep by Mahalanobis distances were shown in Table 3. In the dendogram seen that KG and SC sheep were a group sheep with the closest genetic distance. The two of sheeps (KG and SC) were closer to KS sheep than the BC sheep. Nevertheless the four breeds of sheep (KG, SC, KS, and BC) have close genetic distance and have a farther genetic distance with LG sheep.

In previous studies have reported the genetic distance between the five breeds of sheep by the body size; has showed genetic relationships accurately and high suitability in explaining among the five breeds of sheep (Handiwirawan et al. 2012). Comparison of dendogram built based on the characteristics of voice and body size seen in Figure 3. Position KG sheep was difference between dendogram made by voice characteristics compared dendogram made based on the

body size. Unlike the dendogram built from voice characteristics, based on body size KG sheep has the genetic distance that is closer to the sheep LG and form a separate group with a group of sheep KS, SC and BC.

As described in previous studies by Handiwirawan et al. (2012) although the common ancestor for KS and KG sheep were SC sheep, but the population of SC sheep as common ancestor for KS and KG sheep were a different population. The population of SC sheep as common ancestor for KS sheep was a crossbreed between St. Croix sheep and Local Sumatra sheep, while the population of SC sheep who become common ancestor for KG sheep was a crossbreed between St. Croix sheep and Local Garut sheep. The difference results of Mahalanobis distance values and dendogram showed that environmental factors provide a more powerful influence on the voice characteristics than body size. Voice characteristic was more labile than morphological traits (Mahler & Gil 2009) so that the influences of surrounding environment against voice variables chances were pretty high that caused the differences in results between the two methods. Some environmental effects have been identified affect the characteristics of the voice produced. It has been reported that social isolation and stress environment affect the characteristics of the voice emitted sheep and goats (Engeldal et al. 2012; Siebert et al. 2011). Taylor & Reby (2010) have found that fluctuations in the emotional or motivational physiology affect the voice characteristics. Results of research on birds found that environmental factors that affect the voice was habitat structure, source of noise and weather conditions (Brumm & Naguib 2009). Environmental influences that are not able to be eliminated in this study were the possibility of communication between the sheep that are likely to affect the voice characteristics. Naturally, sheep was a type of livestock that have a congregate character, do not like to be alone. Sampling the voice of sheep in the study was collected by separating the sheep from the group in a separate enclosure so the sheep produced voice for their inconvenience. Nonetheless, communication can still be done by sheep samples that can be done with a combination of visual, auditory and olfactory/chemical transmission except for physical contact.

Nevertheless, the results of voice characteristics analysis showed only slight differences compared with the results of body size analysis, so that this method has a good chance to be applied. If the environmental factors of considerable influence can be eliminated, this method is a method that has the advantage because of not needing to catch or touch the animals observed and easier in its undertaking. The usefulness of this method is great especially for application in wild animals or kept extensively.

## CONCLUSION

Variables for the voice characteristic which can be used as a differentiator of sheep was related to the frequency of voice produced sheep, especially the third quartile frequency, center frequency, maximum frequency and the first quartile time. Dendogram built based on the Mahalanobis distance of voice characteristics put Composite Garut sheep in the group who was less accurate. Slight differences in the grouping of sheep shown from the analysis of voice and body size showed that the genetic distance estimation method using voice characteristics data have the opportunity to apply.

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# Carbohydrate and Protein Digestions on Palm Kernal Cake by Mannanase BS4 and Papain Cocktail Enzymes

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## ABSTRAK.

Rakhmani SIW, Pangestu Y, Sinurat AP, Purwadaria T. 2015. Kecernaan karbohidrat dan protein bungkil inti sawit dengan enzim koktil Mannanase BS4 dan papain. JITV 20(4): 268-274. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1245

Penggunaan enzim mannanase BS4 pada bungkil inti sawit (BIS) dapat meningkatkan energi metabolis. Pencampuran enzim BS4 dengan protease diharapkan dapat meningkatkan kecernaan protein BIS. Penelitian ini bertujuan untuk menentukan proporsi optimal campuran enzim β-mannanase dan protease getah papaya dalam menghidrolisis bungkil inti sawit (BIS). β-Mannanase diproduksi melalui fermentasi substrat padat (FSP) menggunakan bungkil kelapa sebagai substrat. Enzim papain diekstraksi dari getah pepaya (GP) yang diperoleh dari penyadapan getah pada kulit buah pepaya yang belum matang kemudian dikeringkan pada suhu rendah. Evaluasi pencampuran enzim pada BIS dilakukan pada pH 5,8 dan 40°C yang sesuai dengan kondisi lingkungan pencernaan unggas. Perbandingan volume enzim campuran (β-mannanase BS4: papain-v/v) adalah 100 : 0, 75 : 25, 50 : 50, 25 : 75, dan 0 : 100%. Aktivitas *E. javanicum* BS4 β-mannanase adalah 86 $U.ml^{-1}$ dan papain dari getah pepaya (dari KB, DB, DT, dan GP) masing-masing adalah 7, 3, 1, dan 18.000 $U.g^{-1}$. Getah pepaya dipilih sebagai protease dalam campuran enzim dan dibandingkan dengan enzim komersial produksi Merck (CMP) sebagai kontrol positif. Hasil menunjukkan bahwa β-Mannanase BS4 mempunyai aktivitas degradasi karbohidrat, dan aktivitas kecernaan protein tidak terdeteksi. Papain menunjukkan aktivitas degradasi protein, dan tidak mempunyai aktivitas degradasi terhadap karbohidrat. Rasio campuran enzim 50 : 50 menunjukkan aktivitas sinergis tertinggi dalam degradasi protein BIS, terjadi sedikit peningkatan pada produksi asam amino sebagai produk hidrolisis protein PKC, namun gula reduksi yang terbentuk jauh lebih rendah daripada campuran enzim 100 : 0 dan 75 : 25, yang produksi gula reduksinya hampir sama. Dapat disimpulkan campuran enzim yang paling optimum adalah pada perbandingan volume mannanase BS4 dengan getah pepaya adalah 75 : 25, atau masing-masing setara dengan perbandingan aktivitas enzim 14 U : 10 U.

**Kata Kunci**: Mananase BS4, Papain, Campuran Enzim, Bungkil Inti Sawit

## ABSTRACT

Rakhmani SIW, Pangestu Y, Sinurat AP, Purwadaria T. 2015. Carbohydrate and protein digestion on palm kernal cake by Mannanase BS4 and papain cocktail enzymes. JITV 20(4): 268-274. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1245

Supplementation of the mannanase BS4 enzyme on palm kernel cake (PKC) increased its metabolisable energy (ME), and supplementation with protease is expected to increase its protein digestibility. Therefore, the purpose of this research is to determine the optimum proportion of cocktail enzymes between BS4 β-mannanase (produced by *Eupenicillium javanicum*) and protease (papain) and their degradation activities on carbohydrate and protein of PKC. The β-Mannanase was produced by the mold through solid substrate fermentation (SSF) using coconut meal as the substrate. The papain was extracted from papaya latex (PL), collected by longitudinal incisions on unripe papaya fruit and oven dried overnight. The evaluation of enzyme cocktails for PKC hydrolysis was done at pH 5.8 and 40°C which are similar with poultry intestine condition and both enzymes are still active. The β- mannanase BS4 : papain were mixed with some proportions, i.e.: 100 : 0; 75 : 25; 50 : 50; 25 : 75 and 0 : 100% (by volume) in order to study the optimum cocktail composition ratio. The activities of β-mannanase towards gum locust bean was 86 $U.ml^{-1}$ and papains PL activity was 18,000 $U.g^{-1}$respectively. PL was chosen for synergistic reaction and compared with a commercial Merck papain (CMP, 20,000 $U.g^{-1}$) as positive control. β-Mannanase BS4 showed carbohydrate digestion activity, and protein digestion activity was not detected. Papain showed protein digestion activity and no carbohydrate digestion activity. Enzyme cocktails of 50 : 50 from PL protease showed slightly increased in synergistic protein digestion activity in PKC. However, its reduction sugar production was much lower than 100 : 0 and 75 : 25. Amino acids production by enzyme cocktails 75 : 25 were higher than that of 100 : 0. As a result, the best volume composition of β-mannanase BS4 and papaya latex was 75 : 25 (v:v) or 14 : 10 (U:U).

**Key Words**: Mannanase BS4, Papain, Cocktail-Enzymes, Palm Kernel Cake

## INTRODUCTION

World palm oil production dominated by Indonesia and Malaysia wich covered for 80 to 90% of total global palm oil production. In 2014 Indonesia was at the top as oil palm producer with 33 million metric ton and followed by Malaysia (19.8), Thailand (2), Colombia (1.11) and Nigeria (0.93) million metric tons (Index Mundi in 2016). The total area of Indonesia oil palm plantation in 2014 was 8 million hectares and will be growing to 13 million hectares by 2020.

Palm Kernel Cake (PKC) is a by-product of the Palm (*Elaeis guineensis* Jacq.) kernel oil industry. Indonesian PKC production in 2014 was 4550 million MT. Proximate analysis showed that PKC contains crude protein 14-20%, lignin 8-15%, fat 5-11%, crude fiber 13-20% and gross energy 4408 kkal/kg, (Eziezhi et al. 2007; Alimon 2004). In term of the protein content PKC is considered as a good feed ingredient. However, PKC is also high in non- starch polysaccharides (NSP) comprised between 46.6% and 78% (Omar & Hamdan 1998). The high NSP content which is dominated by mannan (78%), cellulose (12%), arabinoxylan (3%), glucuronoxylan (3%) caused the digestibility of PKC poor (Dusterhoft et al. 1991). High content in NSP leads to stickiness in fecal and NSP is also as a trapping matrix for protein and other nutrients. Therefore, the NSP is considered as an anti-nutritional factor in poultry feeding.

In poultry feeding regime, supplementation with exogenous enzymes such as carbohydrase (cellulose), protease and phytase (Ravindran 2013) is a common practice, lately. Enzyme inclusion will minimize the effect of the anti-nutritional factors. Enzyme such as β-Mannanase (endo-1,4-β-mannan mannanohydrolase, EC 3.2.1.78) randomly hydrolyzes β-1,4 mannosidic linkages in mannan, glucomannan, galactomannan and galactoglucomannan into mannose, glucose and galactose (Gilbert et al. 2008). The β- Mannanase BS4 enzyme will degrade mannan link become monosaccharide and resulted in increasing of fiber digestibility and opening the NSP trapping to release the protein. However, the protein digestibility was still considerred low. Supplementation of proteases such as papain will increase protein digestibility. Enzymes cocktail consist of carbohydrase and protease in good proportion will benefit in increasing of nutrient digestibilities of feed ingredients, especially from agricultural by-product such as PKC.

Some enzyme manufacturers have developed enzymes cocktail,. For example, *Novozymes* developed a versatile enzyme cocktail with increased catalytic activity and thermostability by introduction into a *Trichoderma reesei* to produce cellulases mixture, cellobiohydrolase II and beta-glucosidase. Supplementation of the PKC diet with an enzyme complex resulted in a reduction in jejunal contents viscosity (Sundu 2006), improvement in feed conversion in broilers (Chong et al. 2008], and amelioration of the negative effects of feeding a diet containing PKC on the productive performance of laying hens (Soltan 2009).

Utilization of β- mannanase BS4 to increase digestibility of solid heavy phase for poultry diet had been reported (Pasaribu et al. 2009). Solid heavy phase (SHP) is a by-product material of palm oil factory obtained by ceramic filtration from liquid waste. Another report showed that the supplementation of β-mannanase BS4 enzyme to fermented palm kernel cake increased the metabolizable energy of the fermented PKC. Substitution of soy bean meal between 25% and 50% by enzyme-treated fermented PKC did not show any detrimental effect on the performances of laying hens (Sinurat et al. 2014).

The cocktail enzymes of crude β-mannanase from *Eupenecillium javanicum* BS4 and papain of papaya latex was studied and reported here. The objective of this experiment was to determine the optimum proportion of cocktail enzymes between *Eupenicillium javanicum* BS4 β-mannanase and papain and its degradation activity toward carbohydrates and protein of PKC.

## MATERIALS AND METHODS

***E. javanicum*** BS4 was IRIAP collection, coconut kernel cake (CKC) was obtained from Indofeed, palm kernel cake (PKC) from Charoen Pokphand. Green and unripe of papaya fruit was obtained from local market. Papain of *Carica papaya* fruit: Papaya peel (PP), flesh (PF), stalks and leaves (PSL) and latex (PL). Mannanase enzyme BS4 production was carried on a solid state fermentation system at tray incubation system with 100 kg capacity.

### Mannanase production and papain extraction

β-Mannanase produced on solid substrate fermentation was prepared by addition of of 1.5 dosage Mandels mineral solution (Mandel & Reese 1957) to a sterilized coconut meals, and a 10% of a homogenate culture inoculum. The moisture content of the substrate was adjusted to 60%. Incubation for enzyme production was 7 days at 28°C. Crude enzyme was extracted using 0.05 M acetate buffer pH 5.8 at 1 : 10 ratio and ten times concentrated using $(NH4)_2SO4$ precipitation.

Papains were obtained from papaya peel (PP), flesh (PF), stalks and leaves (PSL) macerated in 0.1M phosphate buffer pH 7 (1 : 1). Juices were filtered with a double cloth to obtain crude enzyme. Saturated ammonium sulfate solution was used to precipitate the crude papain of PP, PF and PSL. The pellets were collected after centrifugation at 4°C, 16980 G, for 10 minutes. The latex of the Carica papaya (PL) was

collected by cutting the skin of the unripe fruit, then dried under 60°C and powdered.

### Enzyme activity assay

β-Mannanase activity was determined by using 0.5% Locust Bean Gum (LBG) as the substrate. Reducing sugar was measured by DNS method as described by Miller (19 59). Reactions were carried out in the buffer of 0.2 M Na-acetate at pH 5.8 and incubated at 40°C for 30 minutes, otherwise for determination of optimal pH and temperature. pH and temperature were chosed to mimic the gastrointestinal condition of chicken. One unit of β-mannanase activity is defined as the amount of enzyme that hydrolyzed locust bean gum equal into 1 μmol mannose per minute under the assay condition. The activity was calculated after substraction the mannose production from the samples those were from the reactions of substrate and enzymes with controls those were from the same reactions but without incubation.

For synergistic evaluation the saccharification activity was determined on the PKC substrate, its product reducing sugar was determined as glucose with DNS method. Reactions were also prepared at the same pH and temperature as mannanase activity but incubated for 2 hours. One unit of saccharification activity is defined as the amount of enzyme hydrolyzed the carbohydrate of PKC into 1 μmol glucose per minute under the assay condition. Substraction with the control were also calculated.

Protease activity of papain was assayed by the method of Anson (1938) on casein incubated at 0.2 M phosphate buffer at pH 7 and 37°C. The amino acid produced by the protease was stained with Folin Ciocalteau reagent. One protease unit is defined as that quantity of enzyme that liberates the equivalent of 1 μmol of tyrosine per minute under the condition of the assay.

Enzyme evaluation (single and cocktail) was conducted at pH 5.8 and 40°C. Enzyme optimum condition studies were conducted as followed: β-mannanase BS4 activity was determined in a range of pH 4.5-6.2 and temperature of 40-55°C. Papain activity was determined in a range of pH 4.5-7.5 dan temperature of 40-75°C.

### Synergistic activity of BS4 and papain

The effect of enzymes in carbohydrate digestibility were tested similar to saccharification activity determination of β-mannanase BS4 and protein digestibility similar to protease activity determination of PL papain activity but on PKC instead of casein. Different ratios of β-mannanase BS4 and papain mixtures were prepared in order to determine the optimum ratios to improve the nutrient digestibilities of PKC as shown in Table 1. pH and temperature condition were determined from the optimal condition where two enzymes would be working and similar with poultry gastrointestinal condition. The data were analysed using descriptive analysis.

## RESULTS AND DISCUSSION

Enzymes activities of Papaya Latex (PL) and β-mannanase (BS4) are presented in Table 2. The activity of β-mannanase (BS4) used in this experiment was 86 U/ml and the highest activity of protease was shown in PL papain (18,000 Units/ml). Commercial papain (Merck EC 3.4.22.2) activity was 20,000 Units/ml or g??? under the same assay condition. Papaya latex protease activity up to 30534 Units had been reported (Nitsawang et al 2006). For further study, PL papain was used to make an enzyme cocktail with BS4 β-mannanase.

**Table 1**. Ratio of β-mannanase BS4 and papain in enzyme cocktail

| Enzyme Ratio (% by volume) | |
|---|---|
| Mannanase BS4 | PL protease |
| 100 | 0 |
| 75 | 25 |
| 50 | 50 |
| 25 | 75 |
| 0 | 100 |

**Table 2**. Protease activity of papaya fruit parts and mannanase activity of BS4*

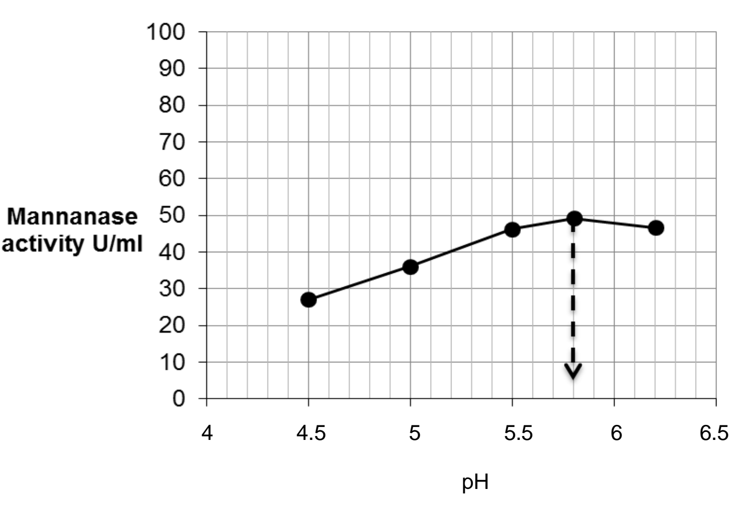
| | Source of Enzyme | |
|---|---|---|
| | Activity (U.ml$^{-1}$) | Activity (U.g$^{-1}$) |
| PP | 7 | ND |
| PF | 3 | ND |
| PSL | 1 | ND |
| PL | ND | 18,000 |
| CMP | ND | 20,000 |
| BS4 | 86 | ND |

*Assay condition: pH 5.8 and 40°C;** Casein as substrate; PL was papaya latex; CMP was commercial papain

The effect of pH and temperature on β-mannanase BS4 activity and for PL-papain were presented in Figure 1 and in Figure 2 respectively. The optimum activity for β-Mannanase was at pH 5.8 and 50°C, and

active in the range of pH 4.5-6.2 and temperature of 40-55°C. Papaya latex papain had an optimum protease activity at pH 6.5 and 70°C and shows activity in the range of pH 4.5-7.5 and 40-75°C. These informations showed that both enzymes will work well in the poultry gastrointestinal condition, even though not reach the optimal activities. The pH of the poultry gastrointestinal condition is laid between pH level of 4.90 Venda (local) chicken and 6.73 of the broiler chickens (Mabelebele et al. 2014). These enzymes were also active in the range of temperature of 40 and 55°C that was closed to the chicken digestive tract temperatures, i.e., 42°C (Dawson & Whittow 2000 *in* Husmaini 2011). The protease optimum temperature and pH activity of PL papain was similar with of commercial papain obtained from Merck (Figure 3), except for the activity in which the commercial papain (20000 U.g$^{-1}$) was higher than the PL papain (18000 U.g$^{-1}$; Table 2). Previous studied reported that papain was active in the range of temperature 60-70°C and pH level of 3 and 9 (Amri & Mamboya 2012). For evaluation the synergistic activity condition reactions were carried out at pH 5.8 and 40°C.

As a common knowledge, enzyme is protein. In designing of enzyme cocktail especially when mixed with protease, the possibility that protease will hydrolyze the companion enzyme should be noted. In this study, both enzymes were subjected for hydrolysis of PKC carbohydrates especially mannan and PKC protein (Figures 3 and 4). It was shown that papaya latex did not digest carbohydrates of the PKC (Figure 3).



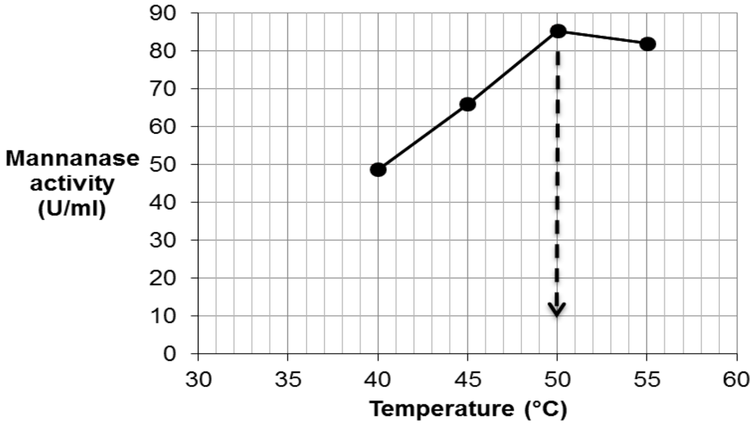




**Figure 1**. The effect of pH and temperature on β-mannanase BS4 activity

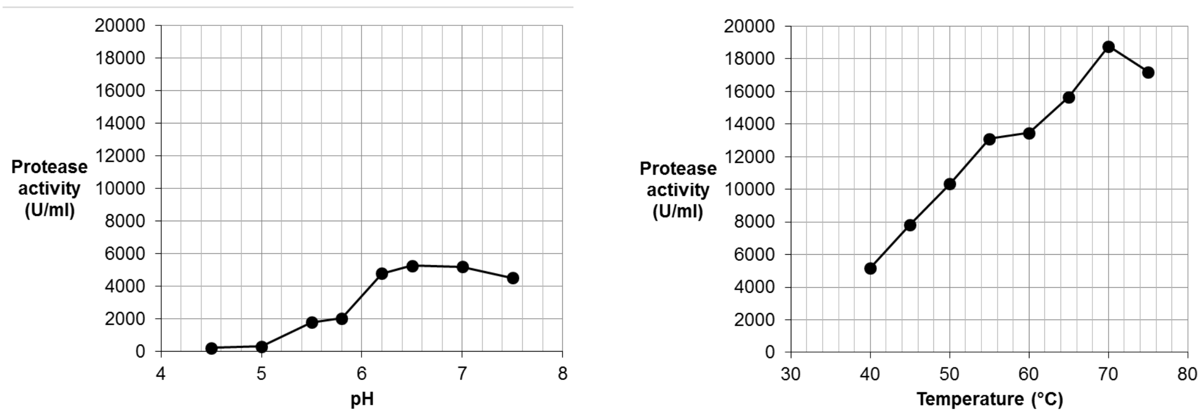



**Figure 2.** The effect of pH and temperature on protease activity of Papaya latex

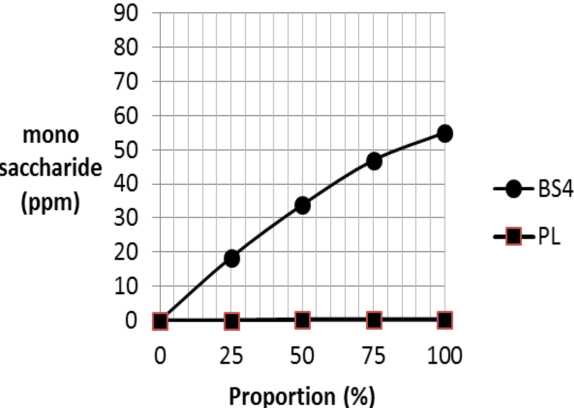



**Figure 3**. Reducing sugars production from hydrolysis of palm kernel cake by proportion mixtures between β-Mannanase BS4 and papaya latex

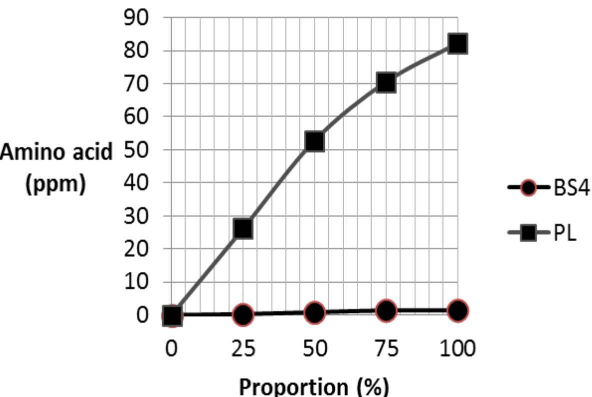



**Figure 4**. Amino acids production from hydrolysis of palm kernel cake by different proportion mixtures between β-mannanase BS4 and papaya latex

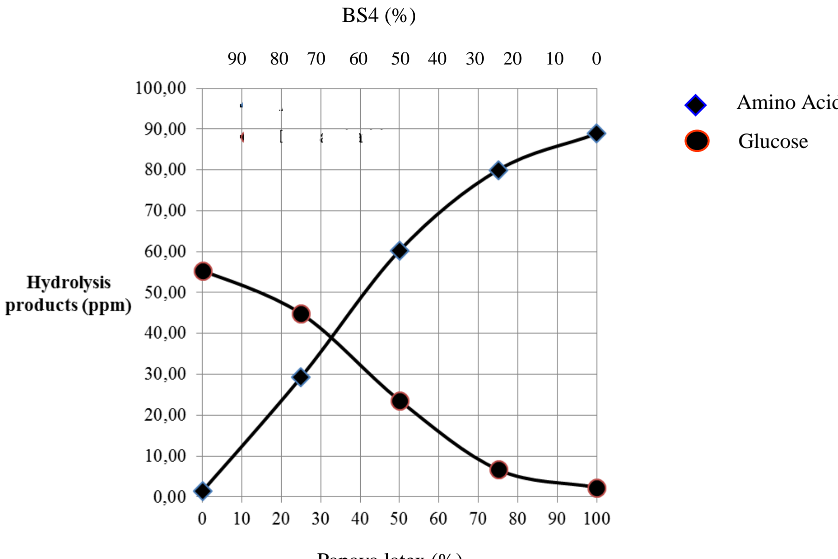



**Figure 5**. Protease and carbohydrase activities of cocktail enzymes with different proportion of Papaya Latex (PL) and mannanase (BS4) in hydrolyzing palm kernel cake

However, crude β-mannanase BS4 enzyme showed slightly protease activity (Figure 4) and liberated the PKC amino acids in a low concentration, i.e., up to 1.5 ppm.

The β-Mannanase BS4 enzyme showed to degrade the carbohydrates and slightly degraded the protein of the PKC. The Papaya latex degraded the protein but did not degrade the carbohydrates of the PKC. The addition of protease increased the value of protein digestibilities. Supplementation of papain alone increased the digestibility of PKC protein. The protein which is trapped in the PKC fiber fraction would be hard to be attacked by the PL protease since it showed a low saccharification activity. Carbohydrase enzyme disrupted the encapsulating effect of the cell wall and released the structure protein (glycoprotein) in soybean meal had been reported (Meng & Slominski 2005). It was assumed that exogenous carbohydrases and protease increase nutrient digestibility as the effect of disruption in cell wall integrity, production of fermentable disaccharides, low-molecular weight polysaccharides and oligosaccharides will then improving protein solubility and digestion (Cowieson & Ravindran 2008). An improvement of 16% protein digestibility in the corn-soybean based diet due to the effect of multi-carbohydrase enzymes addition was also reported (Cowieson 2010).

The volume ratios of cocktail enzymes of mannase BS4 : papaya latex (50 : 50) (Figure 5), showed the increase of amino acids up to 15% when compared to 50% volume papaya latex alone (Figure 4). The PKC protein was released when mannan fiber was degraded by mannanase BS4. The protein and protein-bound carbohydrates or acid detergent insoluble crude protein (ADICP) are digested. In contrast to the production of amino acids, the reducing sugar production as the result of PKC carbohydrates degradation by enzyme mixture of 50 : 50 ratio decreased up to 42% of β-mannanase BS4 alone (Figure 3). The synergistic effect of β-mannanase BS4 and papaya latex in the proportion of 75 : 25 showed increased production of amino acids up to 8% and reduction sugar up to 25%. The optimum cocktail composition was 14 U of β-mannanase BS4 enzyme and 10 U of papaya latex papain. This ratio showed a good synergistic activity between protease and BS4 carbohydrase in producing reducing sugars and amin acids of palm kernel cake.

## CONCLUSION

Optimum activity for β-mannanase at pH 5.8 and 50°C, and active in the range of pH 4.5-6.2 and 40-55°C. Papaya Latex had an optimum protease activity at pH 6.5 and 70°C and of pH 4.5-7.5 and 40-75°C. Both enzymes work well in the poultry gastrointestinal environment, i.e., at pH 5.8 and 40°C. The optimum cocktail composition to digest palm kernel cake was 14 U of β-mannanase BS4 enzyme and 10 U of papaya latex papain protease since it increased both carbohydrates/fiber and the protein digestibilities.

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# Trypanocidals Effectivity against Some Isolates of *Trypanosoma evansi* Propagated in Mice

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## ABSTRAK

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Surra merupakan salah satu penyakit menular pada berbagai jenis hewan yang disebabkan oleh protozoa darah. Surra disebabkan oleh *Trypanosoma evansi* (*T. evansi*) dan seringkali berakibat fatal berupa kematian yang tinggi terutama pada kuda, kerbau dan unta. Salah satu pengendalian penyakit Surra dapat dilakukan dengan pengobatan menggunakan trypanosidal yang efektif. Efektifitas trypanosidal untuk Surra harus didasarkan pada hasil uji sensitivitas. Oleh sebab itu perlu diteliti efektivitas berbagai trypanosidal terhadap beberapa isolat *T. evansi* yang berasal dari beberapa daerah kasus maupun wabah Surra di Indonesia agar diketahui kesesuaian dan efikasinya. Metode pengujian dilakukan dengan desain *pre test-post test*. Mencit DDY diinfeksi dengan beberapa isolat *T. evansi* dari beberapa daerah kasus dan diamati parasitemianya. Setelah parasitemia mencapai puncaknya, maka mencit diobati dengan masing-masing trypanosidal dengan dosis bertingkat. Selanjutnya mencit diamati perubahan parasitemianya selama kurang lebih satu bulan. Hasil penelitian menunjukkan bahwa setiap isolat memiliki kepekaan yang berbeda terhadap trypanosidal sehingga penggunaan trypanosidal cenderung spesifik lokasi. Secara umum, suramin dan *melarsomine dihydrochloride* merupakan dua trypanosidal yang paling efektif untuk beberapa isolat di Indonesia. Adapun *isometamidium chloride* tidak direkomendasikan digunakan untuk pengobatan kasus surra di Indonesia.

**Kata Kunci:** *Trypanosoma evansi*, Trypanosidal, Parasitemia, Surra

## ABSTRACT

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Surra is one of infectious diseases in various types of animals caused by blood parasites called *Trypanosoma evansi*. It is fatally occurred, especially in horse, buffalo and camel. Surra may be controlled by effectively trypanocidals treatment based on the results of its sensitivity test. Therefore, it is necessary to study the effectiveness of various trypanocidal against some *T.evansi* isolates originating from several regions in Indonesia with surra case to determine its suitability and efficacy. The test was carried out by pre-test - post-test. Mice were infected by several *T.evansi* isolates from various infected areas. Their parasitaemia were observed. After reaching peak of parasitemia, the mice were treated by trypanocidal with different doses. Parasitaemia alteration was observed for one month. Observation results showed that all isolates had different sensitivity to the trypanocidals, so that trypanocidals application tended to specific location. Generally, suramin and melarsomine dihydrochloride were the most effective trypanocidal for some Indonesian isolates. In contrast, isometamidium chloride was not recommended to be used for surra control in Indonesia.

**Key Words:** *Trypanosoma evansi*, Trypanocidal, Parasitemia, Surra

## INTRODUCTION

Surra is one of infectious diseases in various species of animals caused by blood parasites called *Trypanosoma evansi* (*T. evansi*). Surra is transmitted by bloodsucking flies (haematophagous flies). Horse and buffalo are sensitive to surra and often leads to high mortality. This happens in Surra outbreak on the island of Sumba, East Nusa Tenggara province in 2010 until 2012. The outbreak resulted in 1159 horses, 600 buffaloes and cows have died (Dirkeswan 2012). Surra still occur in some parts of Indonesia, such as Kalimantan (Borneo), Banten, Lampung, Aceh, and other territories.

One methods for controlling Surra can be done using trypanocidal. Currently, there were 2 approaches, using extract of some herbs and chemically synthetic of active ingredients. Studies using extract of herbs ingredients have been carried out both *in vitro* or *in vivo*. But so far, some herbs extract did not success as anti-*T. evansi* as reported by Abdelrahman (2011), Aman (2013) and Dorneles et al (2013). On the

contrary, Nzelibe et al. (2013) tested trypanocidal activity of *Azadirachta indica* seed extract (NSE) and leaves of *Tridax procumbens* (TP) which showed a success as trypanocidal without relapse when it was combined. Those extracts were not available commercially, and economically those extractions were impossible to be applied to cow, buffalo, and horse.

Therefore, surra treatment using herbs extract still could not be expected, so that must have to rely on chemically synthetic of active ingredient which have been used for 30 to 90 years (Subekti 2014). Active ingredients that have been used as drugs or trypanocidal are *suramin*, *melarsomine dihydrochloride*, *diminazene diaceturate*, *quinapyramine* and *isomethamidium chloride* (Steverding 2010; Melaku & Birasa 2013). Unfortunately, some trypanosome isolates have been reported to develop resistance to some of these trypanocidals from various countries (Melaku & Birasa 2013). Therefore for an effective treatment against Surra should be based on a sensitivity test to trypanocidal (Melaku & Birasa 2013). This is because some trypanosidal may not be appropriate for certain *T.evansi* isolates. Subekti (2014) states that *T.evansi* isolates originating from different regions have different sensitivity to trypanocidal. On the other hand, trypanocidal which is currently marketed in Indonesia is *isometamidium chloride* and *diminazene diaceturate*. The effectiveness of the two drugs are not known against some *T.evansi* isolates in Indonesia that is attacking livestock. Information about the differences in the sensitivity of *T.evansi* isolates of Indonesia against various types of trypanocidal also very limited. Therefore it is important to investigate the effectiveness of various trypanocidal against some *T.evansi* isolates originating from Indonesia, especially from the region of cases or outbreaks of Surra.

## MATERIAL AND METHOD

### *Trypanosoma evansi* isolate

*T.evansi* isolates used in this study were from Sumba Timur, province of East Nusa Tenggara; Serang, province of Banten; Hulu Sungai Utara, province of South Kalimantan; and Pesawaran, province of Lampung. All of isolates were propagated in mice before used for infection and treatment.

### Infection of experimental animals, treatment, and parasitema observation

Female DDY strain mice were acclimated and weighed for body weight (BW) grouping. Mice were divided into 5 groups with 5 mice in each group (Table 1). Each mice were infected by $10^5$ trypanosoma intraperistoneally (Sones et al. 1998). Treatment was carried out when the infected mice have reached 4+ of parasitemia or equivalent to $10^8$-$10^9$ trypanosoma/mL of blood.

Treatment was carried out intraperitoneally using each drugs or trypanocidal and dose applied individually according to mice body weight (Table 1) as described by FAO (Uilenberg 1998). Drug used consisted of *melarsomine dihydrochloride, suramin, diminazene diaceturate* and *isometamidium chloride*. Parasitemia observation was carried out every day for one week post-treatment and continuing every two days in the subsequent observation period up to one month.

**Table 1.** Experimental design of some trypanocidals against different *T.evansi* isolates

| Isolate | *Melarsomine dihydrochloride* (mg/kg BB) | | *Suramin* (mg/kg BB) | | *Diminazene diaceturate* (mg/kg BB) | | *Isometamidium chloride* (mg/kg BB) | |
|---|---|---|---|---|---|---|---|---|
| | 0.25 | 0.75 | 5 | 10 | 3.5 | 7 | 0.5 | 1 |
| 372 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| 373 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| S13 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| S18 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| A14 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| PLS | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

372 = *T.evansi* isolate from Sumba Timur, province of East Nusa Tenggara (isolated in 2012)
373 = *T.evansi* isolate from Sumba Timur, province of East Nusa Tenggara (isolated in 2012)
S13 = *T.evansi* isolate from Serang, province of Banten (isolated in 2014)
S18 = *T.evansi* isolate from Serang, province of Banten (isolated in 2014)
PLS= *T.evansi* isolate from Pesawaran, province of Lampung (isolated in 2013)
A14= *T.evansi* isolate from Hulu Sungai Utara, province of South Kalimantan (isolated in 2014)

Parasitemia observation was carried out with 3 techniques. First by native observation using a microscope. Negative results on the observation of native then followed by observation using MHCT (*Microhaematocrit centrifugation Technique*) and BCT (*Buffy Coat Technique*) as described in the OIE (2012). Mice were declared cured if result of blood test is not found Trypanosome with native examination, MHCT or BCT until the end of the experiment. Otherwise, if the one of those observations was positive, mice were stated sick and parasitemia positive.

## RESULT AND DISCUSSION

### Efficacy of several trypanocidals against several *T.evansi* isolates

Test result showed that anti-trypanosome drug (trypanocidal) had different efficacy to some isolates of *T.evansi* from several regions in Indonesia. Treatment with *melarsomine dihydrochloride* in mice infected with *T.evansi* isolates 372 and 373 have been declared cured at a dose 0.25 and 0.75 mg/kg BW (Figure 1.A). Treatment with *melarsomine dihydrochloride* at a dose of 0.75 mg/kg BW, also provides a satisfactory recovery (100% cured) of the mice infected with *T.evansi* isolate S13, S18 and PLS (Figure 1A). These results are similar to previous studies in mice infected with the 371 and 375 isolates originating from Sumba Timur. In mice that have been infected with the isolate 375, *melarsomine dihydrochloride* can cure mice at a dose of 0.25 and 0.75 mg/kg BW (unpublished data). In contrast, mice that have been infected with the isolate 371, only cured by *melarsomine dihydrochloride* at a dose 0.75 mg/kg BW (unpublished data). The mice infected with the PLS isolate from Lampung only 80% is recovered at a dose of 0.25 mg/kg BW.

*Suramin* effectively used for most isolates of *T.evansi* being tested. *Suramin* at a dose 5 and 10 mg/kg BW can cure 100% of mice infected with the isolates 372, 373, S13, PLS and A14. *Suramin* at a dose 10 mg/kg BW cures only 80% of mice infected with the S18 isolate (Figure 1.B). In mice that had been infected with the isolates 375 and 371, *suramin* give 75% recovery at doses of 5 and 10 mg/kg BW (unpublished data).

On the other hand, *diminazene diaceturate* and *isometamidium chloride* which are widely marketed in Indonesia did not show satisfactory effectiveness compared with *melarsomine dihydrochloride* and *suramin*. *Diminazene diaceturate* only effective for mice infected with the PLS isolate at doses of 3.5 and 7 mg/kg BW. In contrast, mice infected with the 372 and S13 isolates only recovered when treated with diminazene diaceturate at dose of 7 mg/kg BW (Figure 1.C). Conversely, all of mice infected by the sixth *T.evansi* isolates did not cured when treated by *isometamidium chloride*. Generally, mice still showed high parasitemia after treatment. This fact provides evidence that the six *T.evansi* isolates being tested are resistant to treatment with *isometamidium chloride* up to a dose 1 mg/kg BW.

Resistance to *isometamidium chloride* has been widely documented. Among them are reports of Macaraeg et al (2013) which states that a cure can be achieved when using *isometamidium chloride* at a dose 10 mg/kg BW in *T.evansi* isolates from the island of Luzon, and Visayas, Philippines. The dose is 10 times higher than recommended dose as used in this experiment (Subekti 2014). However, Homeida et al. (1980) reported that mice infected with *T.evansi* coming from the East Sudan at a dose of $10^5$ per mice and treated with 10 mg/kg BW of *isometamidium chloride* only partially cured (approximately 40% recovery). Two mice were cured through observations of more than 30 days, 2 mice showed a relapse (40%) and 1 mice died (20%). These results suggest that the usage *isometamidium chloride* treatment more than 1 mg/kg BW also does not guarantee a cure. Jatau et al (2010) reported that treatment with *isometamidium chloride* at a dose of 0.5 mg/kg BW failed to cure mice infected with *T.evansi*. Conversely at a dose 1 mg/kg BW given 5 days post-infection only provide temporary relief followed by relapse at all experimental animals on the eleventh day after the treatment, otherwise if treated at 8 and 11 days post-infection fails to provide relief (Jatau et al., 2010). This condition is likely due to high levels of parasitaemia so the mice die.

Generally, trypanocidal test using *isometamidium chloride* against *T.evansi* isolate from Indonesia showed the same result with Sudan isolate (Homeida et al. 1980) or Luzon and Mindanao Island isolates (Macaraeg et al. 2013) and Jatau et al. (2010). In this study did not use *isometamidium chloride* more than 1 mg/kg BW according to some consideration. First, *isometamidium chloride* is an *ethidium bromide* derivate and has long withdrawal time. Secondly, there is no guarantee of cure by providing higher doses. There is obstacle in its field extrapolation due to its expensive price. One gram/sachet of trypanocidal with active ingredient of *isometamidium chloride* in Indonesia is IDR 250,000. Treatment at a dose of 10 mg/kg BW on large animals with a body weight about 300 kg, needs about 3 grams per head. That means, an animal with a weight of 300 kg takes about 3 sachets so that the cost of drugs per head is IDR 750,000. That cost does not include other cost. It is very expensive and certainly not affordable for farmer in the most Indonesian regions.

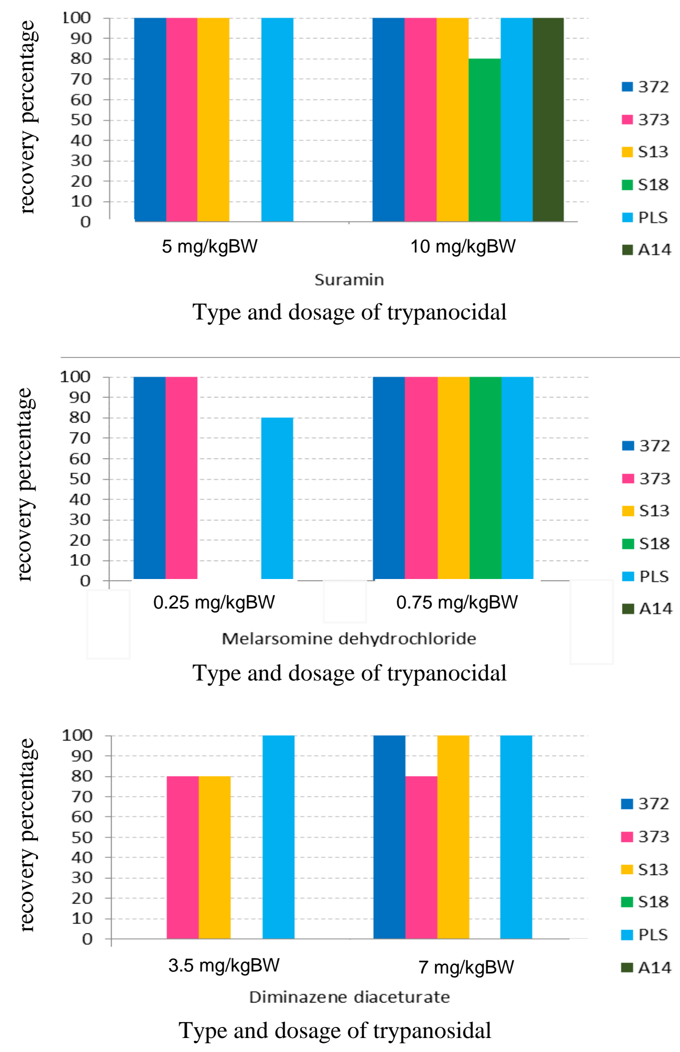


**Figure 1.** The recovery rate of 80% and 100% of mice infected with several isolates of *T. evansi* and treated by several trypanocidals (A) *Melarsomine dihydrochloride*; (B) *Suramin*; (C) *Diminazene diaceturate*
372 = *T.evansi* isolate from East Sumba, East Nusa Tenggara (isolated in 2012)
373 = *T.evansi* isolate from Sumba Timur, East Nusa Tenggara (isolated in 2012)
S13 = *T.evansi* isolate from Serang, Banten (isolated in 2014)
S18 = *T.evansi* isolate from Serang, Banten (isolated in 2014)
PLS= *T.evansi* isolate from Pesawaran, Lampung (isolated in 2013)
A14= *T.evansi* isolate from Hulu Sungai Utara, South Kalimantan (isolated in 2014)

**The phenomenon of relapses in trypanocidal sensitivity test**

*T.evansi* categorized as sensitive to trypanocidal if the infected animal were healed 100% until the end of the experiment. Therefore, trypanocidal at the dose considered to have excellent efficacy. Sometimes can occur in some animal in the experimental group that showed a relapse. Relapse is the occurrence of parasitaemia in mice that have been treated and declared cured on previous observations. The parasitaemia will remain occur until the end of the observation during the experiment or animals die after relapse. The animals were kept showed parasitaemia after treatment with trypanocidal, then the drug considered ineffective and isolates are not sensitive due to the their resistance to the drug. Resistance can occur at certain dose levels or at all dose levels.

The phenomenon of relapse may be linked to several possibilities. First, the drug levels in the blood are not sufficient to kill all parasites. Thus most of the parasites can not come into direct contact with the drug. This possibility is indicated by the presence of most of the animals who relapse within a short time and then disappear again until the end of the experiment. Elimination of parasites after relapse may be caused by

contact with drugs that are still high levels in the blood while the parasites who relapse are very few.

The second possibility is the existence of sub-populations or clones that are resistant. Sub-population was minor population, so that the sub-population will take time to reproduce until it appears as parasitaemia. Indication of the existence of sub-populations of *T.evansi* in one isolate was reported by Subekti et al. (2013) and De-Menezes et al. (2004). In this type, the parasite that relapses generally will not decline again until the end of the experiment. If the therapeutic dose was increased, the parasite will disappear or remain relapse. This evidence indicates that the sub-population who relapse at higher doses is Trypanosome population that has a stronger resistance to the drug.

**Relapse on treatment using *Melarsomine dihydrochloride***

The experimental results demonstrate that treatment with *melarsomine dihydrochloride* shows there are some isolates *T.evansi* who relapse (Figure 2A). Isolate experiencing highest relapse was A14 from Hulu Sungai Utara, South Kalimantan. In isolate A14, 80% was relapse at dose 0.25 mg/kg BW and 50% was relapse at dose 0.75 mg/kg BW. This result indicated that higher dose was needed for isolate A14 treatment.

Isolate S13 and S18 (from Serang, Banten) relapsed by 25% at dose 0.25 mg/kg BW. Similarly, isolate PLS (from Pesawaran, Lampung) relapsed by 20% at dose 0.25 mg/kg BW. *Melarsomine dihydrochloride* at a dose of 0.75 mg/kg BW effective for treatment in 83.3% of *T.evansi* isolates that have been tested, and even provide excellent efficacy to the 372 and 373 isolates from Sumba Timur, as it only requires a dose of 0.25 mg/kg BW.

Relapse in treatment using *melarsomine dihydrochloride* was also reported by Akbar et al. (1998) in Pakistan who infected *T.evansi* into camel. Result showed that *melarsomine dihydrochloride* at dose 0.25 mg/kg BW had cure rate by 66.66%. However, Kabi et al. (2009) reported that there was relapse in isolate *T.evansi* from Uganda in treatment using *melarsomine dihydrochloride* at dose 0.125 mg/kg BW. It also showed that 100 % mice died on $18^{th}$ day infection. However, at dose 0.25 to 1 mg/kg BW, all of mice was cured (Kabi et al. 2009). Two isolates from Sumba Timur (isolate 372 and 373) had similar sensitivity with *T.evansi* isolate from Uganda used by Kabi et al. (2009) which was cure with melarsomine dihydrochloride at dose 0.25 mg/kg BW. The other fourth Indonesian isolates (isolate S13, S18, A14 and PLS) require *melarsomine dihydrochloride* with higher doses than two isolates of Uganda. This is due to recovery of 100% is only achieved when using doses ≥0.75 mg/kg BW.

**Relapse on treatment using *Suramin***

On treatment with *suramin*, *T.evansi* isolates who relapse less than those treated using *melarsomine dihydrochloride* (Figure 2B). These results provide evidence that the sixth isolates of *T.evansi* that have been tested have better sensitivity to *suramin* compared to *melarsomine dihydrochloride*. Generally, the use of *suramin* at a dose 10 mg/kg BW was effective for the treatment of 83.33% *T.evansi* isolates tested. Suramin even provides excellent efficacy in 66.67% of isolates were tested, namely 372, 373, S13 and PLS as it only requires a dose of 5 mg/kg BW. Isolates experiencing relapse were isolate S18 and A14. Isolate A14 was 100% relapsed in treatment using *suramin* at dose 5 mg/kg BW but had no relapse at dose 10 mg/kg BW. In isolate S18, 20% animal treated by *suramin* relapsed at dose 5 mg/kg BW or 10 mg/kg BW. This indicates the possibility of resistance to *suramin* in these isolates.

In Indonesia, some *T.evansi* isolates were resistant to *suramin* at dose 10 mg/kg BW (Payne et al. 1994). Zhou et al. (2004) also reported *T.evansi* isolates from China that were resistant to *suramin* and did not recover at a dose 10 mg/kg BW. Gillingwater et al. (2007) also reported that the STIB 780 and 781 isolate from Kenya were resistant to *suramin*. Korir et al. (2013) said that isolate EATRO 1886 from Busoga, Uganda (*Trypanosoma brucei rhodesiense)* also resistant to *suramin* at 2.5 mg/kg BW. Such evidences showed that resistance to *suramin* was a natural phenomenon.

**Relapse on treatment using *Diminazene diaceturate***

Isolates who relapse after treatment using *diminazene diaceturate* were more higher than *suramin* or *melarsomine dihydrochloride*. At dose 3.5 mg/kg BW, there were 5 isolates relapsed (Figure 2C). Isolate 372 and S13 relapsed by 60% and 20% respectively. Isolate A14, 373, and S18 relapsed at dose 3.5 mg/kg BW and 7 mg/kg BW. These results indicate there were sub-populations that are resistant to *diminazene diaceturate* in stock population of A14, 373 and S18 isolates. Therefore, *diminazene diaceturate* only slightly effective at a dose 7 mg/kg BW (33.33% isolates were recovered), while at a dose of 3.5 mg/kg BW only 16.67% of isolates were recovered.

In Indonesia, *diminazene diaceturate* was given at a dose 3.5 mg/kg BW. Applications of *diminazene diaceturate* with a dose 3.5 mg kg BW for Surra was wrong because the dose is too low despite frequent use in the field (Desquesnes et al. 2013). Although the clinical symptoms disappear but actually a small number of parasites in the blood are still alive and will multiply and cause a relapse in animals (Desquesnes et al. 2013; Gutiérrez et al. 2013). In the event of trypanosomosis caused by *T.b.brucei*, *T.congolense* and

*T.vivax*, *diminazene diaceturate* was recommended at a dose 3.5 to 7 mg/kg BW intramuscularly (Gutiérrez et al. 2013; Desquesnes et al. 2013). Conversely, in the case of surra caused by *T.evansi*, the recommended therapeutic dose was 7 mg/kg BW intramuscularly (Gutiérrez et al. 2013; Desquesnes et al. 2013). Therefore, in Indonesia, when *diminazene aceturate* applied only once treatment will lead to relapse and the case will re-emerge. However, when given twice administration making the accumulative dose being 7 mg/kg BW, is expected to reduce the possibility of relapse even though most of the animals are likely to remain relapse because their resistance to *diminazene diaceturate*.

Akbar et al. (1998) reported that *diminazene diaceturate* at dose 3.5 mg/kg BW cured by 66.66% of infected camels and the rest was relapse and dead. Similar with this study, mice infected with S18 isolates had relapse on 8-10th day post-infection after being treated with *diminazene diaceturate* at a dose of 3.5 mg/kg BW and died at 15-16th day post-infection. Coversely at dose 7 mg/kg BW, there was relapse without followed by death until the end of study. As well as in 373 isolate who showed relapse without followed by death until the end of the study. In contrast to the isolates A14 who relapse after treatment with a dose 3.5 mg/kg BW and followed by death, while at a dose 7 mg/kg BW, 20% relapse and die and the rest (80%) only relapse.

Mohammed (2008) has also been infecting *T.evansi* isolates from Saudi Arabia in Swiss-Webster mice and treated with *diminazene diaceturate* at a dose 3.5 mg/kg BW. The experimental results showed that 60% of mice cured while the rest were died without relapse. These results also indicate that the isolates from Saudi Arabia had a sub-population were resistant to *diminazene diaceturate*. Therefore, 40% of the infected animal does not occur parasitaemia reduction and resulting in death. Qadeer et al. (2015) reported that the goats were infected with *T.evansi* and treated with *diminazene diaceturate* at a dose 3.5 and 7 mg/kg BW have relapse (100%), while the recovery without relapse only occurred at a doses 10.5 mg/kg BW.

Kabi et al. (2009) reported that Swiss Webster mice that have been infected with *T.evansi* isolates from Uganda and treated with *diminazene diaceturate* (dose 1.75 to 14 mg/kg) were all dead on 18th day post-infection. These results correspond with the results of Zhang et al that proves the failure of *diminazene diaceturate* to kill *T.evansi* isolate from China, the Philippines and Ethiopia, both *in vitro* and *in vivo* (Kabi et al. 2009). These results indicate that the *T.evansi* isolates from Uganda used by Kabi et al. (2009) had higher resistance than Saudi Arabia isolate used by Mohammed (2008), Pakistan isolate used by Akbar et al. (1998), and six isolates from Indonesia were used in this study.

Macaraeg et al. (2013) also have infected mice with *T.evansi* isolates of the island of Luzon, Visayas and Mindanao. Luzon isolates require *diminazene diaceturate* with a therapeutic dose of 5 mg/kg BW to cure mice (100%) and failed to cure at lower doses. Visayas isolate require *diminazene diaceturate* at a dose 10 mg/kg BW to achieve 100% recovery, while at doses of 5, 3 and 1 mg/kg BW the recovery rates were 80%, 60% and 0% respectively. *Diminazene diaceturate* at a dose 3 mg/kg was able to cure (100%) mice infected with Mindanao isolates, while at a dose 1 mg/kg BW only cured 20% experimental animal. However, in experiments conducted by Macaraeg et al. (2013) does not provide information whether there is any incidence of relapse.

The results showed that isolate *T. evansi* from different island in Philippine had different sensitivity associated with therapeutic doses are used. *T. evansi* isolates from Luzon and Mindanano were sensitive to *diminazene diaceturate* at dose 5 and 3 mg/kg BW respectively. Visayas isolate require dose above 7 mg/kg BW. The similar result was obtained in experiments using six isolates from Indonesia. Isolates 372 and S13 only recovered 100% when using *diminazene diaceturate* at a dose 7 mg/kg BW. However, four other isolates remained unrecovered at a dose of 7 mg/kg BW and even the S18 and A14 isolates showed relapses (100%).

**Determination of effective Trypanocidal for Surra Disease in Indonesia**

Based on these results, it was difficult to determine the most appropriate trypanocidal used for the treatment of Surra in all regions of Indonesia. This is due to the diversity of the effectiveness of some trypanocidals on different *T.evansi* isolates. Evidence from this study indicates that each isolate has a different sensitivity to the diverse trypanocidal. Those differences require interpretation which leads to a specific location for the use of trypanocidal (Table 2). *T.evansi* originating from different areas will give different responses to the type and dosage of trypanosidal being used. Example from this study, the effective trypanocidal against A14 isolate is *suramin* at a dose 10 mg/kg BW. Instead, the most effective trypanocidal against 372 and 374 isolates are *melarsomine dihydrochloride* at a dose 0.25 to 0.75 mg/kg BW, *suramin* at a dose 5 to 10 mg/kg BW and *diminazene diaceturate* at 7 mg/kg BW. Similar conditions also apply to isolates from other areas also demonstrates different sensitivities.

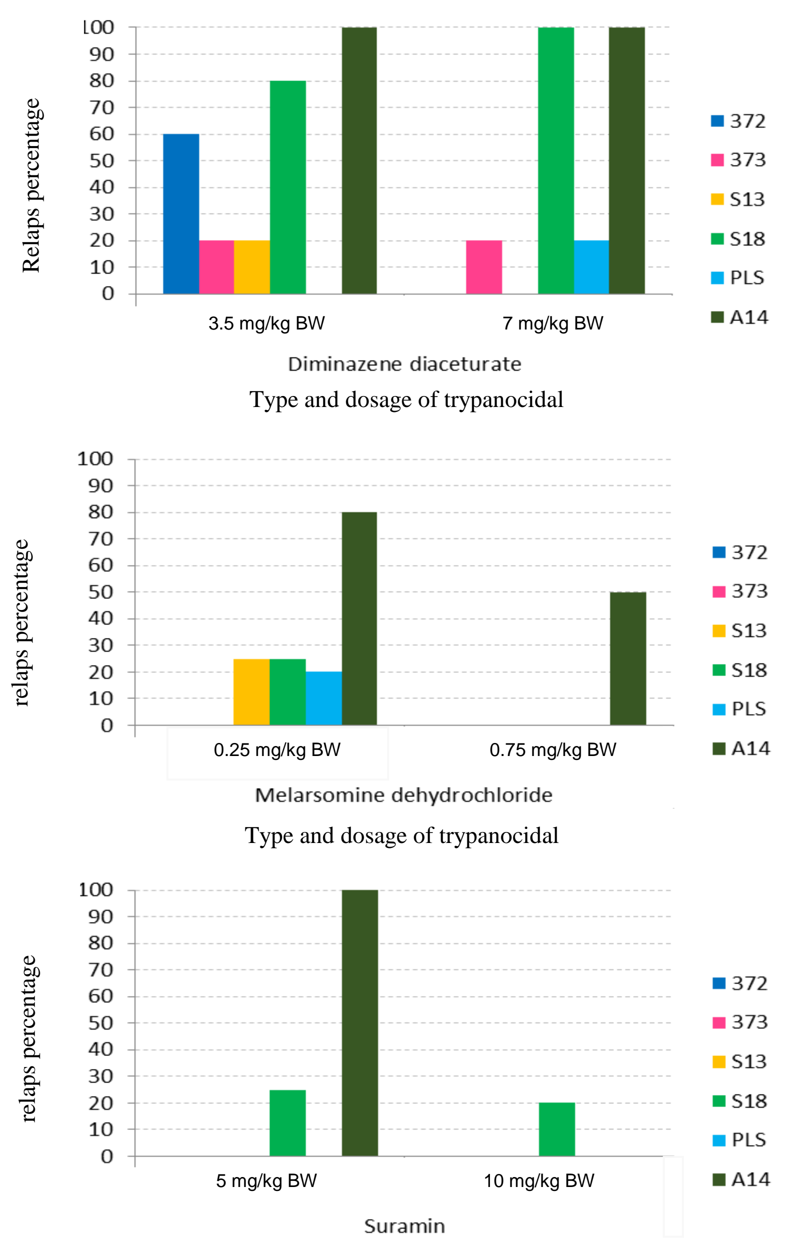


**Figure 2.** Percentage of relapse in treated mice by several trypanocidal. (A) *Melarsomine dihydrochloride*; (B) *Suramin*; (C) *Diminazene diaceturate*

372 = *T.evansi* isolate from Sumba Timur, East Nusa Tenggara (isolated in 2012)
373 = *T. evansi* isolate from Sumba Timur, East Nusa Tenggara (isolated in 2012)
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S18 = *T.evansi* isolate from Serang, Banten (isolated in 2014)
PLS= *T.evansi* isolate from Pesawaran, Lampung (isolated in 2013)
A14= *T.evansi* isolate from Hulu Sungai Utara, South Kalimantan (isolated in 2014)

**Table 2.** Summary of the most suitable selected trypanocidal against *T. evansi* isolate from infected area in Indonesia

| Isolate | Effective drug as Trypanocidal | | |
|---|---|---|---|
| | The Drugs of Choice | The 2nd Choice | Not Recommended |
| 372 | *Melarsomine dihydrochloride, Suramin* | *Diminazene diaceturate* 7 mg/kg BW (100%) | *Isometamidium chloride* |
| 373 | *Melarsomine dihydrochloride, Suramin* | *Diminazene diaceturate* (80%) | *Isometamidium chloride* |
| S13 | - | *Melarsomine dihydrochloride, Suramin, Diminazene diaceturate* | *Isometamidium chloride* |
| S18 | - | *Melarsomine dihydrochloride, Suramin* | *Diminazene diaceturate, Isometamidium chloride* |
| A14 | - | *Suramin* 10 mg/kg BW | *Suramin* 5 mg/kg BW, *Melarsomine dihydrochloride, Diminazene diaceturate, Isometamidium chloride* |
| PLS | *Suramin* | *Melarsomine dihydrochloride* (80-100%), *Diminazene diaceturate* (80-100%) | *Isometamidium chloride* |

**Table 3.** Summary of trypanocidal efficacy criteria against T. evansi isolate from infected area in Indonesia

| Trypanocidal | Dose | 372 | 373 | S13 | S18 | A14 | PLS |
|---|---|---|---|---|---|---|---|
| *Melarsomine dihydrochloride* | 0.25 mg/kg BW | Effective | Effective | Ineffective | Effective | Ineffective | Effective |
| | 0.75 mg/kg BW | Effective | Effective | Effective | Effective | Ineffective | Effective |
| *Suramin* | 5 mg/kg BW | Effective | Effective | Effective | Effective | Ineffective | Effective |
| | 10 mg/kg BW | Effective | Effective | Effective | Effective | Effective | Effective |
| *Diminazene diaceturate* | 3.5 mg/kg BW | Ineffective | Effective | Effective | Ineffective | NA | Effective |
| | 7 mg/kg BW | Effective | Effective | Effective | Ineffective | Ineffective | Effective |
| *Isometamidium chloride* | 0.5 mg/kg BW | Ineffective | Ineffective | Ineffective | Ineffective | Ineffective | Ineffective |
| | 1 mg/kg BW | Ineffective | Ineffective | Ineffective | Ineffective | Ineffective | Ineffective |

NA = not analyzed, it was not analyzed due to the number of live animal was less than 3 heads

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The results of this study indicate that the use of trypanocidal will be divided into three conditions. The first condition is a drug that was not be recommended due to ineffective for infected experimental animals. The second condition is a drug which was only effective in large doses and in effective in small doses. This kind of trypanocidal may still be used regularly followed by an increase in dose and must be accompanied by supervision of the possibility of intoxication. The last condition is drug which was effective used at small doses. Trypanocidal in this category was recommended as the drug of choice for treatment that can be applied effectively.

Nevertheless, drug categories above are only suitable for the treatment of cases of Surra on individual animals and specific locations at the field level. Conversely, when directed as a reference for national policies for the procurement of drugs with the aim of subsidizing procurement of drugs for for a variety of cases in various regions in Indonesia would lead to difficulties in setting priorities for procurement. Therefore, it needs to set priorities by considering the cumulative effectiveness based on the results of the drug test.

Eisler et al. (2001) suggest that a trypanocidal declared effective if the experiment proved ≥ 80% of experimental animals have been recovered. Referring to this provision, it can be arranged a general idea of the effectiveness of some trypanocidal against *T.evansi* isolates from Indonesia that have been tested. Generally, if using criteria of Eisler et al. (2001), *suramin* was effective on all isolates, except on A14 isolate which needed 10 mg/kg BW (Table 3). *Melarsomine dihydrochloride* at dose 0.75 mg/kg BW was effective on 5 isolates (83.33%), except on A14 isolate. *Melarsomine dihydrochloride* at a dose 0.25 mg/kg BW was only effective on 4 isolates (66.7%), except for S13 and A14. *Diminazene diaceturate* declared effective on 4 of 6 isolates (66.67%) were tested when used at dose 7 mg/kg BW and only effective in 3 of 5 isolates (60%) when used at dose 3.5 mg/kg BW. *Isometamidium chloride* was known as a drug that is not effective in all isolates from Indonesia so it is not recommended for use. Therefore, based on these results was known that in general, the order of priority of procurement trypanocidal is *suramin* and *melarsomine dihydrochloride* followed by *diminazene diaceturate* as the last option.

## CONCLUSION

Every isolates have different sensitivities to trypanosidal, hence the use of trypanocidal tend to be specific locations. In general, *suramin* and *melarsomine dihydrochloride* is most effective for some *T.evansi* isolates from Indonesia. The *isometamidium chloride* was not recommended to use for the treatment of Surra in Indonesia.


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# Endemicity of Avian Influenza in Ducks Living Around Commercial Layer Farms

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## ABSTRAK

Tarigan S, Indriani R, Sumarningsih. 2015. Endemisitas penyakit Avian influenza pada itik yang hidup disekitar peternakan ayam petelur komersial. JITV 20(4): 286-296. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1247

Tetua dari semua virus avian influenza adalah itik atau unggas air lainya yang kemudian mengalami mutasi dan adaptasi sehingga menjadi patogen pada ayam atau unggas lainya. Oleh karena itu, penyidikan keberadaan virus influenza pada itik terutama yang dekat dengan peternakan ayam sangat penting. Serum dari 54 ekor itik dan 51 entok yang dipelihara penduduk disekitar peternakan ayam ras petelur komersial di Kabupaten Cianjur dan Sukabumi diambil pada bulan Maret dan April 2014. Indikasi adanya infeksi dilakukan dengan pemeriksaan serologis menggunakan serangkaian alat uji yang meliputi: competitive dan indirect ELISA untuk antibodi nucleoprotein, ELISA MM2e untuk antibodi protein M2e, uji HI, indirect ELISA dan dot blot untuk antibodi haemagglutinin, dan dot blot untuk antibodi neuraminidase. Haemagglutinin rekombinan (H1-H13 dan H15), neuraminidase rekombinan (N1, N2,N7 dan N9) dan rekombinan nucleoprotein virus influenza A digunakan dalam indirect ELISA dan dot blot. Sebanyak 63% dari itik dan 13% dari entok memiliki antibodi terhadap nucleoprotein, dan 62% dari sampel itik yang seropositif nucleoprotein juga memiliki antibodi terhadap M2e. Tingginya seroprevalensi AI pada itik disekitar peternakan ayam ras komersial menunjukkan bahwa penerapan biosekuriti yang ketat pada peternakan ayam komersial masih sangat diperlukan. Berdasarkan hasil pemeriksaan ELISA dan dot blot diduga bahwa pada itik tersebut beredar subtipe H5N2 dan H9N2, selain H5N1. Konfirmasi lebih lanjut dengan isolasi virus perlu dilakukan mengingat subtipe H9N2 dan H5N2 dapat menimbulkan penyakit yang serius pada unggas dan keberadaanya belum pernah diketahui sebelumnya di Indonesia.

**Kata Kunci**: Duck, Immunoassay, Avian Influenza, H5N1, H5N2, H9N2

## ABSTRACT

Tarigan S, Indriani R, Sumarningsih. 2015. Endemicity of avian influenza in ducks living around commercial layer farms. JITV 20(4): 286-296. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1247

The progenitors of all avian influenza viruses are generally derived from ducks or other waterfowl that have undergone mutation and adaptation to become pathogenic in chickens or other poultry. Investigation of the presence of avian influenza viruses in ducks especially those living around chicken farms is, therefore, important. Serum from 54 ducks and 51 Muscovy ducks living around commercial layer farms in the districts of Cianjur and Sukabumi were collected in March - April 2014. The indication of AI-virus infection in those birds was based on an array of serological tests including competitive and indirect ELISAs for antibody to nucleoprotein, MM2e ELISA for antibody to M2e, HI test, ELISAs and dot blot for antibodies to haemagglutinin, and dot blot assay for antibodies to neuraminidase. Recombinant Haemagglutinins (H1-H13 and H15), recombinant neuraminidases (N1, N2, N7 and N9) and recombinant influenza-A nucleoprotein were used in the indirect ELISAs and dot blot assays. As many as 63% of duck samples and 13% Muscovy-duck samples were serologically positive to nucleoprotein, and 62% of the nucleoprotein-seropositive ducks were also positive to M2e. The high seroprevalence of AI in the ducks living around commercial poultry farms suggested that application of strict biosecurity measures on those farms is still needed. Based on the results of the ELISA and dot blot assays, AI virus subtypes H9N2 and H5N2, in addition to H5N1, were suspected to be circulating in those ducks. Further confirmation by virus isolation, however, is required because H9N2 and H5N2 subtypes have yet been unknown Indonesia and both the subtypes can cause serious disease in poultry.

**Key Words**: Duck, Immunoassay, Avian Influenza, H5N1, H5N2, H9N2

## INTRODUCTION

Ducks, including other birds belonging to the orders *Anseriformes* and *Charaddriiformes*, are the natural reservoir of all influenza-A viruses (Alexander 2000). Many of avian influenza-A viruses (AIV) that are pathogenic for domestic chickens originated from low pathogenic AIVs that have undergone mutations in the cleavage site of the haemagglutinin (HA) and reassortment in ducks, before they infect chickens (Guan & Smith 2013). The emergence of a number of HPAI in Southern China, including HPAI H5N1, has been linked with areas populated by ducks and other poultry in high densities (Guan & Smith 2013).

In some countries in South East Asia, domesticated ducks have been proven to be a major risk factor in spreading of HPAI to commercial chickens (Martin et al. 2006; Tiensin et al. 2007). However, a study carried out in Indonesia concluded that ducks were not a major risk factor in the spread of H5N1 in this country. Failure to reveal the association between H5N1 infection in ducks and commercial poultry could be attributed to the fact that the observation was carried out during the lowest grazing activity of ducks that follows the cycle of rice-paddy cultivation (Loth et al. 2011).

The province of West Java in Indonesia is similar to the region in Southern China in regard to the ecology of AIV and intensity and proximity of ducks' and chickens' rearing activities that facilitate the emergence of HPAI (Wan 2012) .

Ducks are raised throughout Indonesia with total population in 2013 estimated at 46,313,000. West Java is the province with the highest population of ducks, 8,943,000 or 19.31%, followed by its neighbor, the Central Java province, with 5,847,000 ducks or 12.63%. Besides ducks, West Java also has the highest number of other poultry (broiler, layer and native chickens) in Indonesia with a total population of 722,738,585 of 1,793,023,090, or 40.31% (DGLAHS 2013).

Our investigation on the occurrence of HPAI in commercial layer farms in West Java, carried out prior to this study, revealed that of eight large farms closely investigated for one year, none was found infected by H5N1 or other subtype of AI viruses. One of the most important sources of infection for those layer farms is presumably the native chickens and ducks living around the farms. Since these extensively raised birds are not normally vaccinated against HPAI, the disease may still be endemic and therefore may become the most important source of infection for the commercial poultry. In our previous study, we reported the presence of ongoing subclinical infection in native chickens. Thirty-six (8.6%) of the 421 chicken tested were positive in either one or more of three serological tests (HI, Influenza-A ELISA and MM2e ELISA) used (Tarigan et al. 2015b). The purpose of this study was to assess the importance of ducks living around commercial farms as the source of AIV infection. In this study we present the examination results of serum samples collected from the same locations in two districts in the province of West Java. In contrast to the layer farms, AIV was found to be endemic in free-range ducks with high seroprevalence.

## MATERIALS AND METHODS

54 ducks and 51 muscovy ducks living near commercial layer farms in Sukabumi and Cianjur districts were bled between March and April 2014. Ducks were collected from 3 villages; Ciwalen (36 ducks), Tangkil Waru (12 ducks) and Tapos (6 ducks), whereas the muscovy ducks were from 7 villages, Ciwalen (17), Cinangka (4), Bedahan (6), Karang Anyer (13), Cipolong (7), Kebun Pedes (2) and Caringin (2). Sample collection was organized and facilitated by the District Animal Health Services. 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 he or she owned, (4) if disease or death in poultry had occurred in the neighborhood, (5) whether they vaccinated their ducks against avian influenza (6) if any of his or her family or neighborhood worked on the commercial layer farms and (7) whether they used to buy culled chicken from the layer farms.

### Haemagglutination inhibition (HI) tests

Haemagglutination test was carried out according to the standard procedures using the haemagglutinating (HA) antigen prepared from a clade 2.3.2 isolate of H5N1 virus (A/Duck/Sukoharjo/Bbvw-1428-9/20012) or a clade 2.1.3 isolate of H5N1 virus (A/Ck/WJ/PWT-WIJ/2006) (OIE 2012). For the HI test, serum to be tested was serially diluted in 25 µl of PBS in V-bottom microtitre plates and an equal volume of HA antigen containing 4 HA units was added. After incubation at 25ºC for 30 min, 25 µl of a 1% suspension of chicken red blood cells was added and incubated for 40 min at 25ºC. The ducks' and muscovy-ducks' sera were treated by adsorption to chicken red blood cells before the HI test. The HI titre was expressed in log2 units of the highest dilution of sera that completely inhibited haemagglutination.

### cNP ELISA

Antibodies to AI virus in collected sera were used as an indication of the presence of AI virus in bird's population. Initially, two serological tests were used; firstly, influenza A or competitive nucleoprotein (cNP) ELISA [Australian Animal Health Laboratory (AAHL), Australia] was used to detect antibody to the NP of influenza-A virus. Testing was carried out according to the protocol provided by the test producer. Secondly, MAP-M2e ELISA was used to detect antibody to external domain of M2 protein of AI (H5N1) virus. The protocols for this test has been described previously (Tarigan et al. 2015a) and used with some variation. Briefly, diluted sera were added to the 96-well microtitre plate that previously had been coated 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 positivity for ducks has not been established, but based on our

previous experiment in layer chicken the cut off OD for positivity was 1.035 x the OD of negative-control serum.

**Indirect-NP (iNP), indirect-H5 (iH5) and indirect haemagglutinins (iHAs) ELISAs**

The iNP and iH5 ELISAs were employed to support the result of cNP and MM2e ELISAs. For these indirect ELISAs, recombinant NP and haemagglutinin H5, obtained from Sinobiologicals Inc. China (Table 1), were used as coating antigens. 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 hours), test serum samples and positive and negative controls, diluted in PBST (PBS pH 7.2, 0.05% Tween-20) at 1 : 100 (or other dilutions when indicated), were added and incubated at 37°C for 1 hour. The negative serum control was collected from a young, AI-free duck, whereas the positive control serum was from a duck that had been vaccinated with an inactive, clade-2.1.3-H5N1 vaccine then challenged with a clade 2.3.2, H5N1 subtype AI virus (A/Duck/Sukohardjo/Bbvw-1428-9/2012). The HI titres of the positive control serum against the challenge virus was 9 log2 and the negative serum control was 0 log2. After washing 4 times with PBST, goat anti-duck-IgG-HRP conjugate (KPL Immunologicals, USA) diluted at 1:100 was added and incubated at 37°C for 1 hr. After washing 4 times, chromogenic (ABTS) substrate was added and the absorbance was recorded using a microtitre-plate reader (Thermo Multiskan Ex).

When samples were positive in NP and MM2e ELISAs, but were negative in H5 ELISA, the type of haemagglutinin (HA) reactive with the sera were determined by iHAs ELISA using similar protocol described for iH5 ELISA and dot blot assay using recombinant proteins of all HA subtypes listed in Table 1. The iNP, iH5 and iHAs ELISAs have not been validated previously and the cut-off values for positivity were unknown. In this study, test results resembled positive or negative serum controls were considered to be positive or negative, respectively.

**Table 1**. Recombinant HA and NA proteins used in this study

| Recombinant protein | Source of HA gene | Catalog No*. |
|---|---|---|
| H1 | A/California/07/2009 (H1N1) | 11085-V08H |
| H2 | A/Japan/305/1957(H2N2) | 11088-V08H |
| H3 | A/Brisbane/10/2007(H3N2) | 11056-V08H |
| H4 | A/Swine/Ontario/01911-1/99(H4N6) | 11706-V08H |
| H5 | A/Indonesia/5/2005(H5N1) | 11060-V08H1 |
| H6 | A/northern shoveler/California/HKWF115/2007(H6N1) | 11723-V08H |
| H7 | A/Netherlands/219/03(H7N1) | 11082-V08B |
| H8 | A/pintail duck/Alberta/114/1979(H8N4) | 11722-V08B |
| H9 | A/chicken/Korea/164/04(H9N8) | 40183-V08B |
| H10 | A/duck/Hong Kong/786/1979(H10N3) | 11693-V08H |
| H11 | A/mallard/Alberta/294/1977(H11N9) | 11704-V08H |
| H12 | A/green-winged teal/ALB/199/1991(H12N5) | 11718-V08H |
| H13 | A/black-headed gull/Netherlands/1/00(H13N8) | 11721-V08H |
| H15 | A/duck/AUS/341/1983(H15N8) | 11720-V08H |
| N1 | A/Hubei/1/2010(H5N1) | 40018-V07H |
| N2 | A/Chicken/Hong Kong/G9/97(H9N2) | 40034-V07H |
| N7 | A/Netherlands/219/2003(H7N7) | 40202-V07H |
| N9 | A/Anhui/1/2013(H7N9) | 40108-V07H |
| NP | A/California/07/2009(H1N1) | 11675-V08B |

*Sinobiologicals Inc. China

**Dotblot and SDS PAGE**

Three microliters of recombinant HA or NA proteins diluted at 20 µg/ml in PBS, were spotted onto a nitrocellulose membrane strip. After air-drying, the membrane was blocked with skimmed milk (5 mg/ml, 2 hours), 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, goat anti-duck-IgG-HRP conjugate (KPL immunologicals, USA) diluted at 1:100 was added then incubated at 25°C for 2 hours. After washing 4 times, chromogenic (DAB) substrate was added to probe antibody bound to the nucleoprotein.

Recombinant proteins were separated in the 10% -acrylamide-separating gels on SDS PAGE. Each recombinant haemagglutinin was dissolved in SDS-PAGE sample buffer at 200 µg/ml, heated in boiled water for 5 minutes and loaded into the SDS PAGE gels 5 µl/3.4-mm-wide well. Proteins in the gel were stained with routine Coomassie blue.

## RESULTS AND DISCUSSION

**Ducks**

All the 54 duck sera were negative in HI test using a 2.3.2 clade H5N1 isolate (A/Duck/Sukoharjo/Bbvw-1428-9/20012) (Hi titre <3 log 2). Other tests used in this study, however, show a high proportion of the sera to be positive to AI. The duck sera could be classified into 6 groups based on combination of OD of MM2e ELISA (low <0.25, moderate 0.25-0.5 and high >0.5) and the results of cNP ELISA (negative and positive) (Table 2).

Only 11 sera (20%), which were negative in cNP ELISA and MM2e-ELISA (MM2e-ELISA's OD<0.25), were considered to be true negative for AI, or at least for H5N1. These results were supported by iNP and iH5 ELISA. When 8 of the 11 sera were tested with the iNP and iH5 ELISAs, all of them were negative as they had OD comparable to that of negative control serum (Figure 1, 2 yellow bars).

Twenty-one sera (39%) were supposedly positive for AI because they were both positive in cNP ELISA and MM2e-ELISA (MM2e-ELISA's OD>0.25). When 8 of the 21 sera were tested with iNP ELISA, 7 sera were positive because they had OD, which were higher than that of the negative control serum (Figure 2, red bars). As a matter of fact, the ODs of some of these sera were even higher than that of positive control serum. The majority of the NP-positive sera were also positive for H5 haemagglutinin because 6 of the 8 NP-positive sera were positive in i-H5 ELISA (Figure 2, red bars). Two ducks of this group (#109 and #118), which were negative in the iH5 ELISA had probably been infected by a non-H5 subtype of AI virus.

The assumption that duck no 109 and 118 were not infected by subtype H5 but by other subtype of AI virus was support by the dot blot assay (Figure 3). The reliability of the assay was affirmed by its results on control sera. As expected, the negative control serum did not recognized any of the recombinant haemagglutinin whereas positive control serum which was derived from duck vaccinated and challenged with a H5N1 virus recognised strongly H5 haemagglutinin with some cross reaction with H2 haemagglutinin. In line with the indirect H5 ELISA, sera from duck 109 and 118 did not recognize the H5 haemagglutinin in dot blot assay (Figure 3). Serum from duck 118 only recognised H9 haemagglutinin, whereas serum from duck 109 regonised H7, H8, H9 and H10 haemagglutinins, but the most prominent reaction was with H9 haemagglutinin. The results of this dot blot assay was in line with the indirect ELISA in which all

**Table 2**. Results of MM2e and cNP ELISAs on 54 sera collected from ducks living near commercial layers farms

| | | cNP ELISA | | | | Total |
|---|---|---|---|---|---|---|
| | | Negative | | Positive | | |
| MM2e-ELISA OD | <0.25 | Serum #: 103, 121, 122, 124, 129, 130, 131, 347, 349, 352, 522 | 11* | Serum #:97, 99, 101, 102, 113, 117, 119, 120, 125, 132, 348, 355, 525 | 13* | 24 |
| | 0.25-0.5 | Serum #:100, 128, 350, 351, 353, 521, 523 | 7* | Serum #:105, 106, 107, 108, 110, 111, 114, 115, 116, 123, 127, 356, 526 | 13* | 20 |
| | >0.5 | Serum #:104, 524 | 2* | Serum #:98, 109, 112, 118, 126, 345, 346, 354 | 8* | 10 |
| | | Total | 20 | | 34 | 54 |

(*) number of ducks in the group

the recombinant haemagglutinins were used to coat the microtitre plate. The highest ELISA OD in this ELISA was found with the H9 haemagglutinin, followed by H8, H10 and H7 haemagglutinins (Figure 4). All recombinant haemagglutinins used in these immunoblot assay and ELISA had high purity and contained the same protein concentration as indicated by the SDS PAGE analysis (Figure 3B). Therefore, non-specific reaction between contaminated proteins and immunoglobulin contained in the duck sera, and 'background noise' due to uneven concentration of haemagglutinins in the assays could be neglected.

There were nine ducks that were negative in iNP ELISA, seven of which had moderate MM2e-ELISA's OD (0.25-0.5) and two had high (>0.5) (Table 2). Further analysis with iNP and iH5 ELISAs on some sera of this group, duck 104 (MM2e-ELISA' OD=2.698), duck 524 (MM2e-ELISA' OD= 0.627) and duck 351 (MM2e-ELISA' OD= 0.401), revealed that the all sera were negative in both iNP and iH5 ELISAs (Figures 2 and 3, blue bars). Because the ducks were seronegative to NP protein based on cNP and iNP ELISAs, the ducks were likely to be seronegative to AI virus. The results of MM2e ELISA for those ducks, therefore, were considered to be non-specific.

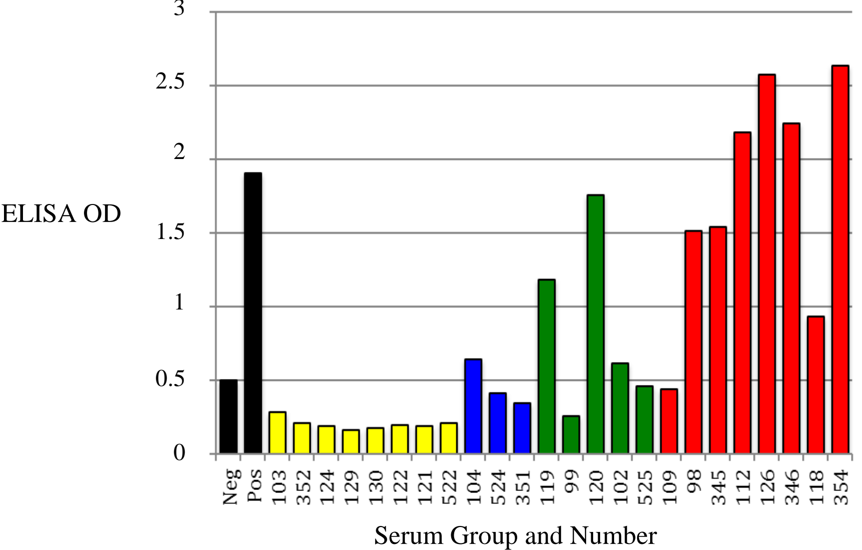



**Figure 1**. Antibody level, indicated by ELISA OD, in duck sera to Influenza-A nucleoprotein determined by iNP ELISA

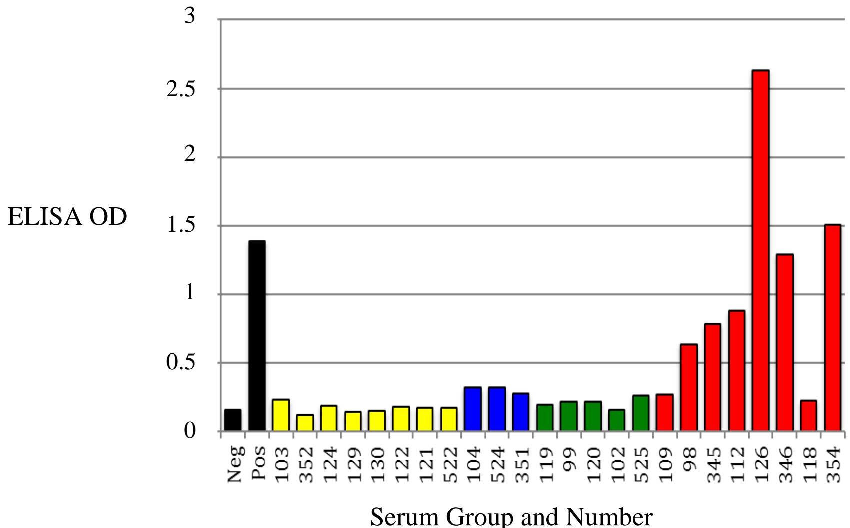
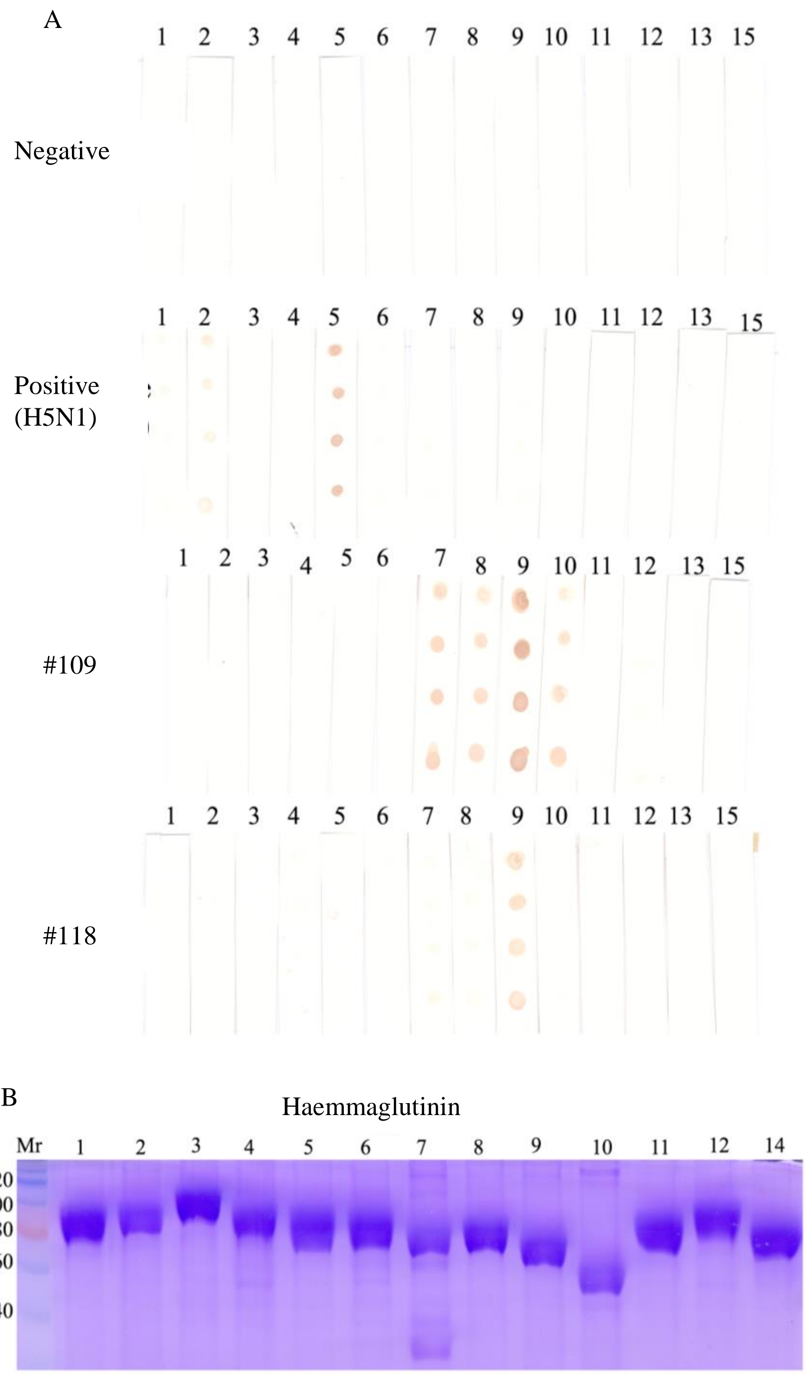



**Figure 2**. Antibody level, indicated by ELISA OD, in duck sera to influenza haemagglutinin H5 determined by iH5 ELISA




(A). Suspension of each recombinant haemagglutinin (3 µl) at 20, 10, 5, and 2.5 µg/ml were spotted onto nitrocellulose membrane strips then reacted with duck sera at 1:400 dilution

(B). Coomassie-blue-stained SDS PAGE of recombinant haemagglutinin to show that all haemagglutinins were pure and had equal concentration

Numbers above the strips and SDS-PAGE gels are the haemagglutinin type; 1, 2, .,..... ., 14 denote haemagglutinins H1, H2, H3, ........ H14

**Figure 3**. Determination of haemagglutinin type recognised by duck sera

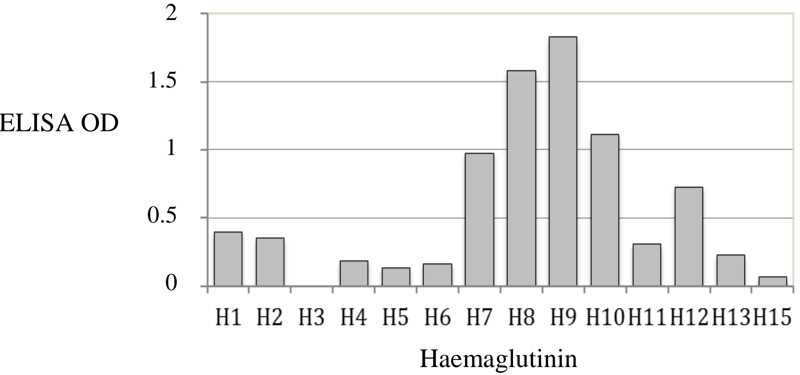
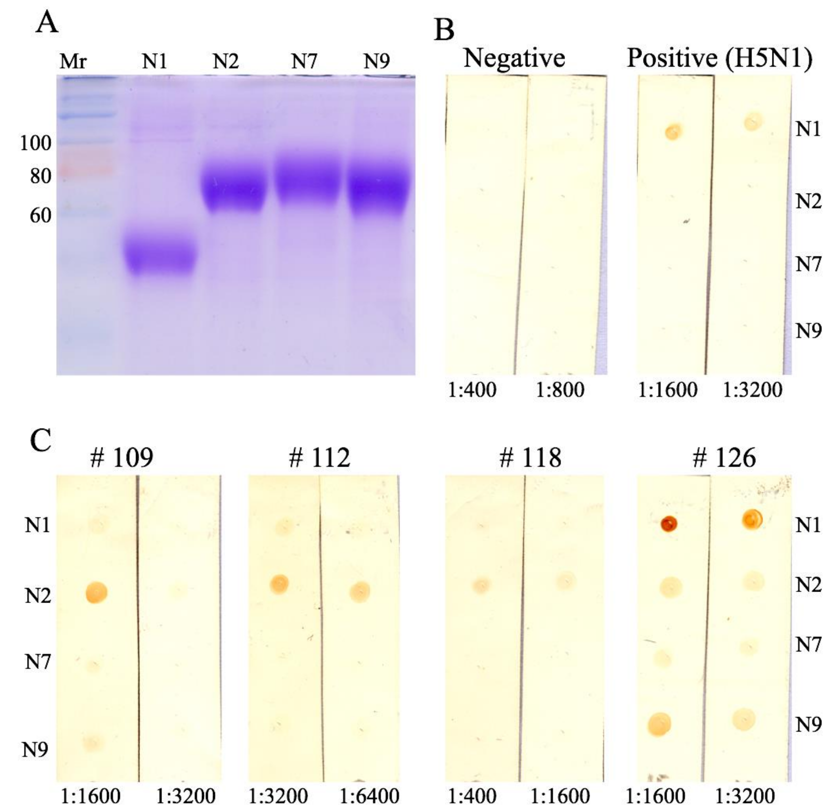



**Figure 4**. Haemagglutinin types recognised by serum from duck #109 in indirect ELISA. Confirmation the result of dot blot (Figure 3). A microtitre plate was coated with each recombinant haemagglutinin (2.5 µg/ml) then reacted with serum from duck #109 at 1:200 dilution




(A). Coomassie-blue-stained SDS PAGE of recombinant neuraminidase N1, N2, N7 and N9 to show that all neuraminidase preparations were pure and in equal concentration. Suspension of each recombinant neuraminidase (3 µl, 20µg/ml) were spotted onto nitrocellulose membrane strips then reacted with duck sera at dilution indicated below the strips

(B). Negative and positive control duck sera

(C). Duck serum samples

**Figure 5.** Neuraminidase types recognised by duck sera

Positive test results in cNP ELISA but negative in MM2e ELISA were found in 13 (24%) sera (Table 3). When five of them were tested in iNP ELISA, only two sera had OD that were higher than that of control negative serum (Figure 2, green bars). Examination with iH5 ELISA indicated that none of the 5 sera examined was positive for H5 haemagglutinin.

**Table 3**. Seropositivity in sampled Muscovy duck against Avian Influenza

| Village | District | No. sera | Positive cNP ELISA | |
|---|---|---|---|---|
| | | | No | Percent |
| Ciwalen | Cianjur | 17 | 3 | 18% |
| Cinangka | Cianjur | 4 | 0 | 0% |
| Bedahan | Cianjur | 6 | 0 | 0% |
| Karang Anyer | Cianjur | 13 | 4 | 31% |
| Cipolang | Cianjur | 7 | 0 | 0% |
| Sasagara | Sukabumi | 2 | 1 | 50% |
| Cibaringbing | Sukabumi | 2 | 0 | 0% |
| Total | | 51 | 8 | 16% |

Reactivity some of the sera to neuraminidase N1, N2, N7 and N9 is presented in Figure 5. Four ducks tested were all positive in cNP and MM2e ELISAs but had variable OD in iH5 ELISA. Duck 126 had high, duck 112 had moderate and ducks 109 and 118 had low, as low as negative control duck, iH5 ELISA's OD. Sera from duck 109, 112 and 118 reacted prominently with neuraminidase N2 whereas that from duck 126 withneuraminidase N1. The dot blot assay considered being reliable since the positive and negative sera control reacted as expected. The positive control serum, which was derived from duck that had been vaccinated and challenged with H5N1 virus, reacted only with the N1 protein, and the negative serum reacted with none of the neuraminidases. (Figure 5B). In addition, the recombinant neuraminidases, based on SDS PAGE, had high purity and even concentration (Figure 3B).

Taken together with previous haemagglutinin assay, ducks 109 and 118 might have been infected with a sub-type H9N2 AI virus because their sera recognized recombinant haemagglutinin H9 and neuraminidase N2. Whereas, duck 112 might have been infected with a sub-type H5N2 AI virus because its sera recognized recombinant haemagglutinin H5 and neuraminidase N2.

**Muscovy ducks**

As compared with ducks, seropositivity for avian influenza in muscovy ducks was lower. Only 8 of 51 (16%) were positive in cNP ELISA (Table 3). The subtype of AI virus responsible for the seropositivity was not assayed as that for ducks. However, on HI test using a clade 2.1.3 isolate of H5N1 virus (A/Ck/WJ/PWT-WIJ/2006) as antigen, four sera were found positive, one serum from Ciwalen village (HI titre 4 log2), and 3 sera from Karang Anyer village (HI titre 5log2, 3log2, 3log2).

**Discussion**

Since it first reported in 2003 until 2008, H5N1 AI was endemic with high incidence among native chicken and ducks in West Java, especially in the districts of Cianjur and Sukabumi (Yupiana et al. 2010). Since then, the number of cases declined gradually until the outbreak of clade 2.3.2 H5N1 in duck in 2012 (Dharmayanti et al. 2014). At the time of sample collection, and a couple of years previously, no report of H5N1 outbreak in the Districts of Sukabumi and Cianjur (Districts' PDSR, personal communication).

Despite the absence of report on the outbreak of HPAI H5N1, the present study shows that subclinical avian influenza is still endemic and common among ducks and Muscovy ducks living around commercial layer farms in the Sukabumi and Cianjur Districts, West Java. The prevalence of infection in those birds seems to be very high as 63% and 16% of sampled ducks and Muscovy ducks respectively were seropositive for AI. This is in contrast to the situation in AI-vaccinated, commercial layer farms in the area, where no AIV infection was recorded for the last 12 months (S. Tarigan personal observation). Further of interest was, that native chickens which were not vaccinated against AI and scavenging together with the ducks had much lower seroprevalence of AI (Tarigan et al. 2015b).

The purpose of this study was to analyze whether the absence of AI infection in the commercial poultry farms correlated with that ducks scavenging around the commercial farms shown previously to be negative for H5N1. Because the population of ducks living around the farms was small, the number of serum samples examined in this study were also small. The samples, therefore, were not representative of ducks in the Cianjur and Sukabumi Districts. Although the number of serum samples was small, the sera were examined thoroughly employing many serological tests. Antibody

to NP, commonly used as a marker for AIV infection, was initially detected with cNP ELISA. The cNP 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 iNP ELISA, although has not been validated previously, has high agreement with the the cNP ELISA. This is not surprising because the indirect ELISA used recombinant nucleoprotein with high purity as the coating antigen.

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. 2015a). This MM2e ELISA has not been validated for used in ducks. However, based on the present study and previous study, this MM2e ELISA could also be adapted for use in ducks (Lambrecht et al. 2007). Most sera with high OD in MM2e ELISA were positive in cNP ELISA and those with low OD in MM2e ELISA were negative in cNP ELISA. Our previous study in layer chicken revealed that the MM2e ELISA was highly specific for identifying chicken infected with H5N1 virus. In the present study, however, the MM2e ELISA might not be as specific as in layer chicken, as two sera derived from non-infected ducks, based on cNP ELISA, had high OD in MM2e ELISA. This false positive, the cause of which was unknown, suggest that the MM2e ELISA is still need to be adjusted and validated for use in ducks. The finding that some ducks were serologically positive for nucleoprotein but had low MM2e-ELISA's OD was not unexpected. The same incident has been observed in chicken and ducks in previous studies (Lambrecht et al. 2007; Kim et al. 2010; Hemmatzadeh et al. 2013; Tarigan et al. 2015a). The nucleoprotein is likely to be more antigenic than M2e because the nucleoprotein, which is made up of 498 amino acid residues, is much bigger than M2e which contains only 23 amino acid, and therefore antibody to nucleoprotein is likely to have longer life (Huddleston & Brownlee 1982; Neirynck et al. 1999).

It was unexpected that all duck sera were negative on HI test using the antigen prepared from a H5N1 AI virus subtype (HI titre of <3 log2) in the present study. Since the iH5 ELISA and immunoblot assay indicated the presence antibody to haemagglutinin in some of the sera, the HI test used in this study may not be sensitive enough to detect the presence of the antibody. The lack of sensitivity may be caused by the unmatched antigenicity between the H5N1 subtype used in the HI test and the virus infecting the ducks.

Although the number of serum samples was small, results obtained in this study were important. First, the seroprevalence of AI in ducks in the vicinity of big commercial layer farms was very high. Even though the virus seems to cause only subclinical infection in those ducks, the AIV may undergo antigenic drift and shift that become pathogenic for chicken. The spill over of LPAI viruses from ducks into poultry and mutate into HPAI viruses has been documented for a number occasions (Swayne 2007). The HPAI H5N1 which was originated from a LPAI underwent mutation in duck in Guangdong province before it spread to chickens (Wan 2012). Secondly, the present study also indicated that AI virus subtype circulating in the duck population was not only H5N1 but also probably subtypes H9N2 and H5N2. Since the later subtypes have never been identified previously in Indonesia, this serological evidence is still inadequate to claim that those subtypes are present in this country. Confirmation of this serological evidence by meticulous effort to isolate the AI virus subtypes from ducks is required because H5N2 and H9N2 are subtypes that cause great economic loss in poultry industry in many country (Lee et al. 2005; Okamatsu et al. 2007; Woo & Park 2008). In addition, human often contracted the H9N2 subtypes leading to serious disease (Cameron et al. 2000; Lin et al. 2000; Matrosovich et al. 2001).

Since the outbreak of H5N1 AI in Indonesia in 2003, the H5N1 AI virus subtype has been known to be the only subtype circulating among ducks in Indonesia, and no other AIV subtype has been identified (Henning et al. 2010). However, since ducks are the natural reservoir of AIV, circulation of other AIV subtypes in this bird is probably common (Alexander 2000). The circulation of AIV subtypes in ducks other than H5N1 has been reported in Bangladesh where H5N1 is also endemic. Based on a survey conducted in in 2009 to 2012, a seroprevalence of 39.76% for AI in semi scavenging ducks was reported, and extremely low percentage (0.09%) of those AI-positive sera were reactive to H5N1 subtype (Khatun et al. 2013). Similarly, a survey carried out in 2009 in Vietnam, where H5N1 was also endemic, identified 22 AI viruses consisting 21 samples H6N1 and 1 sample H9N2 subtypes among 1488 duck's swab samples, and none of the sample positive for H5N1 virus (Hotta et al. 2012).

Infection with the clades 2.1.1 and 2.1.3 of the H5N1 subtype virus results in mild or subclinical disease in ducks, whereas infection with the clade 2.3.2 usually causes severe disease with high mortality in young ducks (Wibawa et al. 2013; Dharmayanti et al. 2014; Wibawa et al. 2014). This later clade caused 223,042 death in ducks at the peak of the outbreak in September - November 2012 (Ditjennak 2013).

The indication that AI virus subtypes, other than H5N1, are circulating among ducks in Indonesia was provided by previous study (Susanti et al. 2008). This study aiming at identifying AI viruses by a PCR technique in the cloacal swabs collected from ducks,

Muscovy ducks and geese in Bogor and Sukabumi Districts, West Java found 21 of 460 samples (4.6%) were positive for H5N1, 13 samples (2.8%) for HxN1, 3 samples (0.7%) for H5Nx and 8 samples (1.7%) for HxNx. Several decades previously, (Ronohardjo 1982) studied avian influenza in ducks in Indonesia and reported that H4N6 and H4N2 were the only subtypes found and the subtypes caused clinical disease in ducks characterised by sinusitis, air sacculitis and poor growth in growing ducks. The samples from which the H4N6 and H4N2 AI virus subtypes isolated were collected from West Java and other places in Indonesia. In this present study, however, serum sample reactive to the recombinant haemagglutinin H4 was not found. In Vietnam, H3N2, H3N8, H4N6, H5N1, H5N2, H6N1, H9N2, H9N6, H11N3 and H11N9 subtypes have been isolated from ducks (Nguyen et al. 2009; Hotta et al. 2012; Nomura et al. 2012)

Both H9N2 and H5N2 are the AIV subtypes that cause great economic losses to the poultry industry and found in many countries. Subtype H5N2 is known to have highly and low pathogenic variants. The high pathogenic variant has been reported to cause severe outbreaks in the USA (Clement et al. 2015), South Africa (Abolnik et al. 2012) and Mexico (Villareal & Flores 1997).

Subtype H9N2 has spread globally and is reported to be enzootic in many Asian countries including China (Zhu et al. 2013; Wang et al. 2014), Korea (Kim et al. 2006; Lee et al. 2011), Pakistan (Cameron et al. 2000), Iran (Ghaniei et al. 2013) and Israel (Banet-Noach et al. 2007). Although subtype H9N2 AIV is classified as LPAI, the economic losses associated with this subtype are enormous in many countries (Jakhesara et al. 2014; Shehata et al. 2015).

Since the H9N2 subtype is widely present in Asia, it is not surprising if the subtype also present in Indonesia. The Asian H9N2 which now has adapted to chicken originally derived from ducks because this subtype was only isolated from duck before 1992 (Guo et al. 2000). This means that the H9N2 that apparently still confine to ducks as observed in this study may one day jump to chickens.

The seroprevalence of AI in Muscovy ducks as found in this study was much lower than that in ducks. It is unknown whether the lower seroprevalence in Muscovy ducks is related to its genetically being less susceptible to AI, or else. Different pathological and immunological responses in Muscovy duck and Peking ducks after challenge with an isolate of H5N1 virus have been described previously (Cagle et al. 2011). In this study attempt to identify AI virus subtype reacting to the sera of Muscovy ducks was not made because the difficulty to obtain anti-muscovy-duck conjugate.

## CONCLUSION

This study showed that based on serological examinations ducks living near commercial layer farms in Sukabumi and Cianjur, West Java are infected subclinically with AIV with high prevalence. Based on reactivity of the duck sera to recombinant haemagglutinins and neuraminidases in indirect ELISA and dot blot assays, subtypes H5N2 and H9N2, in addition to H5N1, were suspected to be present in the duck population. Further study, however, is required to confirm the presence of H9N2 and H5N2 subtypes in Indonesia by virus isolation. Although at the time of sample collection most of the infection was subclinical and confines only to ducks, the AIV may undergo mutation in ducks to become pathogenic for, and spread to chicken.

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# Molecular Identification Technique of *Trypanosoma evansi* by Multiplex Polymerase Chain Reaction

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## ABSTRAK

Sawitri DH, Wardhana AH, Wibowo H, Sadikin M, Ekawasti F. 2015. Pengembangan teknik identifikasi molekuler *Trypanosoma evansi* dengan *Polimerase Chain Reaction* Multipleks. JITV 20(4): 297-307. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1248

*Trypanosoma evansi* adalah parasit hemoflagella yang menginfeksi ternak dan dikenal sebagai penyebab Surra. Beberapa spesies trypanosoma lainnya pada hewan mamalia adalah *T. equiperdum, T. b. rhodesiense, T. b. gambiense, T. vivax, T. congolense, T.theileri*. Beberapa spesies tersebut cukup sulit untuk dibedakan secara morfologi dengan *T. evansi* melalui teknik konvensional (preparat ulas darah). Teknik molekuler dengan polymerase chain reaction (PCR) dilaporkan memiliki kemampuan yang akurat dalam mengidentifikasi, mengkarakterisasi dan mendiagnosis Trypanosoma. Namun demikian PCR tunggal yang digunakan adalah relatif mahal karena setidaknya diperlukan dua atau lebih pasang primer untuk menentukan spesies *T. evansi*. Tujuan penelitian ini adalah untuk mengembangkan teknik identifikasi spesies *T. evansi* dengan PCR multipleks/mPCR (tiga jenis pasang primer dalam satu reaksi) sehingga dibutuhkan waktu yang realtif cepat dan murah. Sebanyak 31 isolat *T.evansi* yang diperoleh dari Bblitvet Culture Collection (BCC) dan Departemen Parasitologi BBLitvet digunakan pada penelitian ini. Isolat-isolat tersebut mewakili isolat yang berasal dari daerah endemis dan Wabah Surra yang diisolasi tahun 1988-2014. Ekstraksi DNA dilakukan pada setiap sampel, termasuk isolat Bang 87 yang telah dimurnikan sebagai kontrol positif. Primer yang digunakan adalah spesifik untuk *T. evansi*, yaitu ITS-1, Ro Tat 1,2 VSG dan ESAG 6/7. Sebelum melakukan mPCR, masing-masing primer dioptimasi dengan menggunakan PCR tunggal. Hasil penelitian menunjukkan bahwa ketiga primer tersebut dapat dikombinasikan dalam satu reaksi dengan teknik mPCR dan mengamplifikasi masing-masing fragmen DNA target dengan sempurna, sehingga 31 isolat teridentifikasi sebagai *T. evansi*. Teknik ini dapat diaplikasikan di lapang dengan biaya yang lebih murah dan waktu yang lebih cepat.

**Kata Kunci**: *Trypanosoma evansi*, Identifikasi, Multipleks PCR

## ABSTRACT

Sawitri DH, Wardhana AH, Wibowo H, Sadikin M, Ekawasti F. 2015. Molecular Identification Technique of *Trypanosoma evansi* by Multiplex Polymerase Chain Reaction. JITV 20(4): 297-307. DOI: http://dx.doi.org/10.14334/jitv.v20i4.1248

*Trypanosoma evansi* is a Hemoflagella parasite that infects cattle and is known as the agents of Surra. Several other trypanosome species infects mammals: *T. equiperdum, T. b. rhodesiense, T. b. gambiense, T. vivax, T. congolense, T.theileri*. Some of these species is quite difficult to be distinguished morphologically with *T. evansi* through conventional techniques (thin blood smear). Molecular technique by polymerase chain reaction (PCR) is reported to have the ability to identify, characterize and diagnose trypanosomes accurately. However, a single PCR used is relatively expensive because it takes at least two or more pairs of primers to determine *T. evansi*. The purpose of this study is to develop *T. evansi* species identification techniques by multiplex PCR/mPCR (the three pairs of primer in one reaction) that takes the relatively fast and inexpensive. A total of 31 isolates *T.evansi* were obtained from Bblitvet Culture Collection (BCC) and the Department of Parasitology BBLitvet used in this study. Isolates represent isolates from endemic areas and Surra outbrake isolated from 1988-2014. DNA extraction performed on each sample, including Bang 87 isolates which has been purified as a positive control. Primers used are specific for *T. evansi*, the ITS-1, Ro Tat 1.2 VSG and ESAG 6/7. Before running mPCR, each primer is optimized by using a single PCR. The results showed that the three primers can be combined in a single reaction with mPCR technique and amplify each DNA fragment target perfectly, so identified 31 isolates as *T. evansi*. This technique can be applied in the field with a lower cost and faster time.

**Key Words**: *Trypanosoma evansi*, Identification, Multiplex PCR

## INTRODUCTION

Surra, a wasting disease in livestock, is caused by hemoflagellate parasite *T. evansi* which transmitted mechanically by haematophagus flies (Herrera et al. 2004; Fernandez et al. 2009). This parasite affecting a wide range of wild species and livestock population (Mulumbu 2006). This disease has the widest

geographical distribution among all pathogenic parasitic species prevalent in Africa, Asia and Central and South America (Devila et al. 2003; OIE 2012).

There are two main types of trypanosoma: the first is transmitted through the saliva/vector bites (salivarian) and the second is transmitted through the vector feces (stercorarian). There are four subgenus trypanosoma salivarian attacking mammals: subgenus Trypanozoon (*T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense, T. evansi, T. equiperdum*); subgenus Dutonella (*T. vivax, T. Uniforme*); sub genus Nanomonas (*T. congolense, T. simiae, T. godfreyi*) and subgenus Pycomonas (*T. suiz*) (Mulumbu 2006). While stercorian trypanosome there is one species (*T. theileri*, subfamily Triatominae) that attack livestock.

Some specieses had similar shape, so that it was difficult to be distinguished morphologically. So far, only *T. evansi* reported attacked livestock animal in Indonesia (Ditjennak 2012). *T. theileri* also sometimes found in cattle and buffaloes in Indonesia (Bblitvet unpublished data). Nevertheless *T. theileri* reported non-pathogenic.

*T. evansi* infection is a prevalent disease that causes considerable economic losses due to weakness, abortion in pregnant animals, estrus cycle disorders, weight loss, decreased productivity and reproductivity, high treatment cost and death (Reid 2002; Jittapalapong et al. 2009). Trypanosome infections also cause immunosuppression effects which triggering to other diseases (Jittapalapong et al. 2009). Directorat General of Livestock reported that in 2010-2012, Surra outbreak attacked 4268 head livestock and 1760 out of them were dead (Ditjennak 2012).

Trypanosome identification, generally was performed based on microscopic observation (morphology, morphometric and parasite motility within the host tissue), host range, and geographical region. Further identification was also based on presence of the parasite in certain organs of vector cycle and ability of the parasite to grow in vivo (in rodents or vector) and invitro (Hoare 1972).

This conventional identification by using thin blood smear and microhematocrit centrifugation test (MHCT) has limitations. Its success depended on the number of parasite on sample observed. Parasite species had similar morphology, so that it was hard to be distinguished (Uilenberg 1998). Masake et al. (2002) states that the diagnosis of trypanosomiasis would have problem if only one or two parasites have found on preparations of thin blood smear with low quality. This may cause misidentified of the trypanosome species. Therefore, accurate species identification was needed to distinguish trypanosome species infecting animals.

Polymerase chain reaction (PCR) technique was reported had accurate ability in identifying, characterizing, and diagnosing trypanosomiasis (Holland et al. 2001; Desquesnes & Dávila 2002). This assay has high sensitivity and specificity to detect 1-10 trypanosoma/ml of blood (Davila et al. 2003) and able to distinguish between species (Desquesnes et al. 2001). Some moleculer markers have been widely constructed to detect, differentiate, and study trypanosome species such as Internal Transcriber Spacer-1 (ITS-1) and Rhode Trypanozoon Antigen Type 1.2 VSG gene (Ro Tat-1,2 VSG) (Salim et al. 2011; Urakawa et al. 2001). According to Salim et al. (2011), PCR ITS-1 product length specificaly was corellated to each trypanosome species. So that can be used as a basic to distinguish the species. Besides, Urakawa et al. (2001) states that one of T. evansi's characteristics was gene encoding Ro Tat 1.2 VSG (Rode Trypanozoon Antigen type 1.2 VSG), which was able to distinguish T. evansi and another Trypanosome specieses. Another moleculer marker was Expression-site-associated gene 6/7 (ESAG 6/7 gene) encoding trafferin receptor of *T. evansi*. It was specific and had high sensitivity (Shahzad et al. 2010). Until now, identification of *T. evansi* species was carried out by using single PCR of ITS-1 and Ro Tat 1.2 VSG primer and was never reported using multiplex PCR technique.

Multiplex PCR technique (mPCR) was developed in 1988 by Chamberlain et al. (1988) and reported to be highly effective for detecting various types of the disease agent in one reaction. This technique is more economical than a single PCR technique for use less chemical reagent in the process of DNA fragments amplification (Batra et al. 2013). Ekawasti et al. (2014) has used mPCR technique to detect *T. evansi* from haematophagus flies (Tabanus sp, Stomoxys sp and Hipobosca sp) as vector of *T. evansi*. The study only used two primers (ITS-1 dan Ro Tat 1.2 VSG) and success to be amplified perfectly on positive samples consisting *T. evansi*.

The aim of this study was to develop mPCR technique using three pairs of primer, the ITS-1, Ro Tat 1.2 VSG and ESAG6/7. The three pairs of primer used will improve the accuracy for the identification and detection of *T. evansi* species. Besides, also able to detect the presence of other trypanosomes species (mix infection of trypanosomes) so that Surra diagnostic in the field can run faster, cheaper and have high specificity and sensitivity.

## MATERIAL AND METHODS

### Parasite source

Thirty one *T. evansi* samples used in this study. Fifhteen isolate sampels were from BBlitvet Culture Collection (BCC) which was collected during 1988-2008. Another isolate samples source was from Departement of Parasitology of BBlitvet which was a

circulating isolates (fifteen isolates was collected from outbrake area during 2012-2014 and one isolate from endemic area in 2013). Those isolates were from 14 locations from 8 provinces (Table 2). Bang87 isolate (from BCC) was used as a positive control. *T. evansi* derived from BCC and circulating isolates in 2012 (Sumba) was stored cryopreservation. While *T. evansi* circulating isolates in 2013-2014 which collected from buffalo blood with Surra positive (Pandeglang) stored in eppendorf tubes at -20°C. Cryopreservation as stabilate through the stages of passage in mice before being stored. We chose 31 isolates based on the availability in BCC and the origin of the isolates representing endemic and outbreaks areas in Indonesia.

**Table 1**. *T. evansi* isolates used in this research

| Code isolate | Code BCC | Isolate origin (sub district, district, Province) | Year | Animal origin | Description |
|---|---|---|---|---|---|
| Bang 87 | P0176 | Bangkalan, Bangkalan, East Java | 1988 | Buffalo | BCC |
| Bang 85 | P0162 | Burneh,Bangkalan, East Java | 1988 | Buffalo | BCC |
| Pml 287 | P232 | Pemalang, Pemalang, Central Java | 1996 | Buffalo | BCC |
| Pml 291 | P233 | Taman, Pemalang, Central Java | 1997 | Buffalo | BCC |
| Sbw 340 | P202 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1998 | Buffalo | BCC |
| Sbw 341 | P203 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1998 | Buffalo | BCC |
| Sbw 363 | P213 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1999 | Buffalo | BCC |
| Sbw 364 | P030 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1999 | Buffalo | BCC |
| Sbw 366 | P029 | Lape Lopok, Sumbawa Besar, West Nusa Tenggara | 1999 | Buffalo | BCC |
| Smi 68 | P169 | Surade,Sukabumi, West Java | 1985 | Buffalo | BCC |
| Smi 369 | P125 | Surade, Sukabumi, West Java | 2008 | Buffalo | BCC |
| Bwi 218 | P075 | Kalibaru, Banyuwangi, East Java | 1992 | Buffalo | BCC |
| Bwi 222 | P076 | Kalibaru, Banyuwangi, East Java | 1992 | Buffalo | BCC |
| Bwi 228 | P077 | Kalibaru, Banyuwangi, Jawa Timur | 1992 | Buffalo | BCC |
| Ash129 | P192 | Tanjung Muda, Asahan, North Sumatera | 1990 | Buffalo | BCC |
| Ash 133 | P178 | Limapuluh, Asahan, North Sumatera | 1990 | Buffalo | BCC |
| Smb 370 | - | Wajelo, East Sumba, East Nusa Tenggara | 2012 | Buffalo | Outbreak, Dept Parasitology |
| Smb 371 | - | Wajelo, East Sumba, East Nusa Tenggara | 2012 | Buffalo | Outbreak, Dept Parasitology |
| Smb 372 | - | Wajelo, East Sumba, East Nusa Tenggara | 2012 | Buffalo | Outbreak, Dept Parasitology |
| Smb 373 | - | Wajelo, East Sumba, East Nusa Tenggara | 2012 | Buffalo | Outbreak, Dept Parasitology |
| Smb 374 | - | Wajelo, East Sumba, East Nusa Tenggara | 2012 | Buffalo | Outbreak, Dept Parasitology |
| Smb 375 | - | Wajelo, East Sumba, East Nusa Tenggara | 2012 | Buffalo | Outbreak, Dept Parasitology |
| Lbk 376 | - | Lebak, Banten | 2013 | Buffalo | Outbreak, Dept Parasitology |
| Munt377 | - | Muntilan, Central Java | 2013 | Buffalo | Endemic, Dept. Parasitology |
| Pdg 378 | - | Cisata, Pandeglang, Banten | 2013 | Buffalo | Outbreak, Dept Parasitology |
| Pdg 379 | - | Cisata, Pandeglang, Banten | 2013 | Buffalo | Outbreak, Dept Parasitology |
| Pdg 380 | - | Cisata, Pandeglang, Banten | 2013 | Buffalo | Outbreak, Dept Parasitology |
| Pdg 381 | - | Cisata, Pandeglang, Banten | 2013 | Buffalo | Outbreak, Dept Parasitology |
| Pdg 382 | - | Carita, Pandeglang, Banten | 2014 | Buffalo | Outbreak, Dept Parasitology |
| Pdg 384 | - | Carita, Pandeglang, Banten | 2014 | Buffalo | Outbreak, Dept Parasitology |
| Pdg 386 | - | Cisata,Pandeglang, Banten | 2014 | Buffalo | Outbreak, Dept Parasitology |
| Pdg 388 | - | Cisata, Pandeglang, Banten | 2014 | Buffalo | Outbreak, Dept Parasitology |

**Table 2**. Primers Sequence used for the amplification single and multiplex PCR

| Primers | | Primer sequences | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| ITS1 | *F* | 5`-CCGGAAGTTCACCGATATTG-3` | 480 | (Njiru et al. 2005) |
| | *R* | 5`-TGCTGCGTTCTTCAACGAA-3` | | |
| RoTat 1.2 VSG | F | 5`-CTGAAGAGGTTGGAAATGGAGAAG-3` | 151 | (Salim et al. 2011) |
| | *R* | 5`-GTTTCGGTGGTTCTGTTGTTGTTA-3` | | |
| ESAG 6/7 | *F* | 5'-CATTCCAGCAGGAGTTGGAGG-3' | 740 | (Isobe et al. 2003) |
| | *R* | 5'-TTGTTCACTCACTC TCTCTTTGACAG-3' | | |

F = primer *forward;* R = primer *reverse*

**Table 3**. Chemical Composition of mPCR optimalisation

| Chemical composition | X 1 reaction |
|---|---|
| dd$H_2O$ | 5.35 µl |
| 5x KAPA 2G Buffer A | 5 µl |
| KAPA 2G Fast DNA Polymerase (5units/ µl) | 0.15 µl |
| dNTPmix (10µM/dNTP) | 0.5 µl |
| ESAG6/7-*Forward* (10µM) | 2 µl |
| ESAG6/7-*Reverse* (10µM) | 2 µl |
| ITS-1-*Forward* (10µM) | 2 µl |
| ITS-1-*Reverse* (10µM) | 2 µl |
| RoTat 1,2 VSG-*Forward*(10µM) | 2 µl |
| RoTat 1,2 VSG-*Reverse*(10µM) | 2 µl |
| Cetakan DNA (100 ng/µl) | 2 µl |
| Total Reaction | 25µl |

## DNA extraction

*T. evansi* from stabilates and buffalo blood was thawed at room temperature. Total genomic of 31 samples was extracted from 100 µl of stabilate/buffalo blood by using Genomic DNA Mini Kit (Geneaid, Taiwan) according to the manufacturer's instructions. Purified DNA was stored at -20°C until further analysis. Samples *T. evansi* from stabilate were coded Mc (mice) and samples from buffalo blood were coded Buf (buffalo).

## PCR Primers

Three primer pairs (1st BASE, Singapore) were used for single and multiplex PCR analysis (Table 1). ITS-1 primers was amplifying DNA at 480 bp fragment length, whereas RoTat 1.2 VSG and ESAG 6/7 primers were amplifying DNA at 151 bp and 740 bp fragment length, respectively (Isobe et al. 2003; Njiru et al. 2005; Salim et al. 2011).

## Bang87 *T. evansi* isolate purification as positive control

Bang87 *T. evansi* isolate (BCC collection) was used as positive control of *T. evansi* species (Sawitri 2016). Bang 87 stabilate was thawed at room temperature and was injected to a mouse using 1 ml Tuberculin syringe. Parasitemia was checked every two days by wet blood smear. At the highest parasitemia ($10^8$ cells/ml) which was usually in the 4-5th day of injection, blood was withdrawn from euthanized mouse by cardiac punctured. An anion exchange column (DE 52 DEAE cellulose) was used to purify parasite from the blood cells according to the method of described by OIE. (2012). The eluent with infect protozoa was collected and proceeding to DNA extraction.

### Single PCR analysis of ITS-1, Ro Tat 1,2 VSG and ESAG6/7 primers for *T. evansi*

First step of mPCR development was optimizing of each primer (Table 1) separately by using Bang87 *T. evansi* isolate as positive control. The PCR products should correspond to the size of the fragment gene of interest.

PCR amplification was performed using KAPA2G ™ Fast PCR kit (KAPA BIOSYSTEMS, USA) on ABi GeneAmp thermal cycler 9700. Each reaction contained a final volume of 25µl, including 2 µl of 50-100 ng of genomic DNA; 5 µl of 5X KAPA 2G buffer A; 0.1 µl KAPA 2G Fast DNA Polymerase (5 unit/ µl); 0.5 µl dNTPmix (10µM/dNTP); 2 µl of each forward and reverse primer (10µM/µl) and 12,4 µl of sterile distilled water. PCR amplification was carried out the following conditions 35 cycles: one cycles initial denaturation step at 95°C for 1 minute; 35 cycles denaturation at 95°C for 10 second; 35 cycles annealing at 58°C for 15 second; 35 cycles extension at 72°C for 15 second and one cycle final extension at 72°C for 10 minutes.

### Multiplex PCR

Multiplex PCR was conducted by combining 3 primers on one PCR reaction. Multiplex PCR was carried out by using modified KAPA 2G ™ Fast PCR kit (KAPA BIOSYSTEMS, USA). Composition of reagent, primer, and template was presented in Table 3. PCR condition was similar with previous step.

### Visualization of PCR product

PCR products were resolved by electrophoresis at 100 volt in 1.5% (w/v) agarose gels stained with SYBR® Safe gel staining (Invitrogen™).Visualization and analysis of fractionated DNA bands were carried out on GelDoc Transluminator (Cleaver). Diagnosis was considered positive when a specific product of each gene was amplified by PCR.

## RESULT AND DISCUSSION

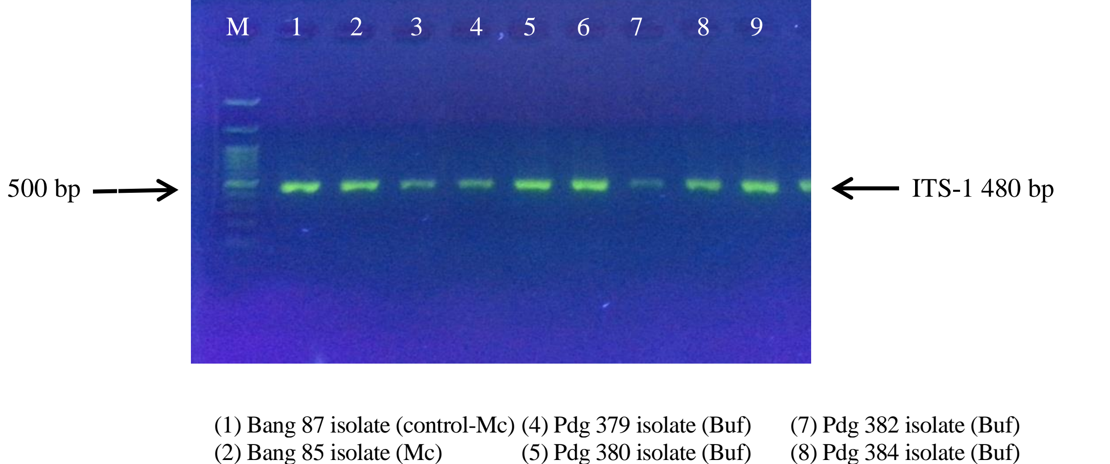
### Identification of *T. evansi* by single PCR

Recent development in the molecular techniques have had considerable input into trypanosome identification, characterisation and diagnosis, accuracy and reliability at various taxonomic levels (Desquesnes & Davila 2002). PCR based methods was widely applied to detect trypanosome with high sensitivity and specificity (Gibson 2009). PCR use to detect DNA trypanosome was a reliable and accurate technique available to identify infected animal species naturally for most species and subspecies of trypanosome (Welburn et al. 2001; Njiokou et al. 2004).

Product of single PCR amplification on Bang87 isolate as positive control with the three primers showed three DNA fragment with different sizes (Figure 1, 2, 3). The first DNA fragment at 480 bp (Figure 1) was an ITS-1 amplicon (Figure 1) (Salim et al. 2011) The second and the third DNA fragment at 151 bp (Figure 2) and 740 bp (Figure 3) was amplicon product of Ro Tat 1.2 (Njiru et al. 2005) dan ESAG6/7 (Isobe et al. 2003) respectively. Another 31 isolates used in this study both DNA template extracted from stabilate (Mc) or buffalo blood (Buf) also produced the same amplicon length (Figure 1, 2, 3). Thus all isolates used in this study was the *T. evansi.* Amplicons quality differences caused by differences in the quality of the DNA template.The three primers amplifies DNA target with the same PCR condition: one cycle of initial denaturation at 95°C for 3 minutes; 35 cycles of denaturation at 95°C for 10 second; 35 cycles of annealing at 58°C for 15 second; 35 cycles of DNA extension at 72°C for 15 second and one cycles of final DNA extension at 72°C for 10 minutes.

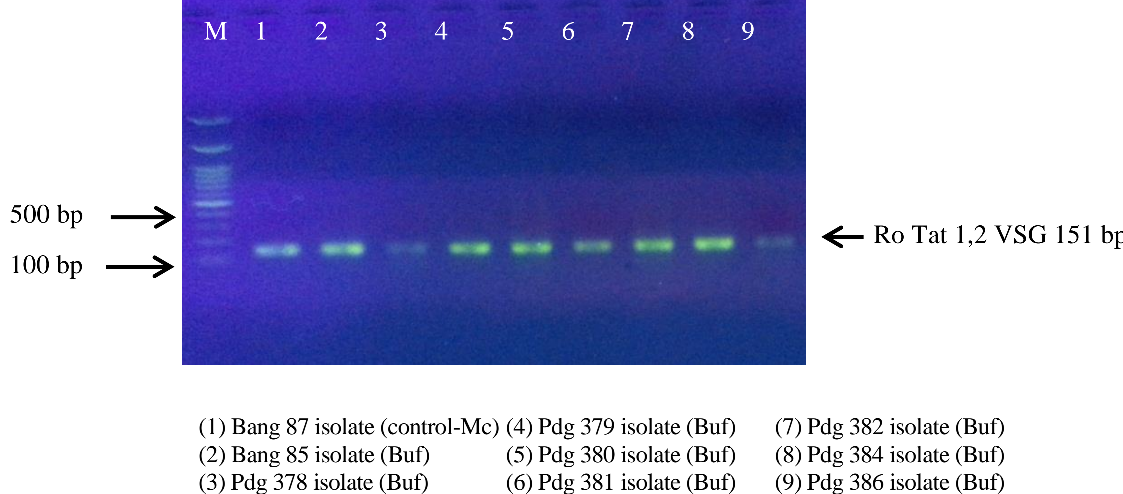
ITS-1 Primer which amplifying internal transcriber spacer-1 Ribosomal RNA (rRNA) gene was reported able to identify some trypanosome due to various length for specific species (Desquesnes & Dávila 2002). Internal Transcriber Spacers (ITS) was lied between repeated sequens at the core of 18S, 5.8S and 28S genes encoding the ribosomal RNA subunits, occurs in approximately 100-200 copies per genome of a trypanosome (Desquesnes et al. 2001). rRNA ITS-1 and ITS-2 sequence were separated by 5.8 S gene and connected by a small and large sub-unit rRNA gene in almost all eucaryotic organism (Hernandez et al. 1993). Internal transcribed spacer regions (ITS) which relatively short size and connected with highly conserved segment become the primer attachment site on PCR process (Desquesnes et al. 2001). The ITS1 is usually 300–800 bp in length, and has a variable length depending on the Kinetoplastida species, but is presumed to be constant within a species. Various ITS segment length between species and interspecies made region ITS a very useful molecular marker to identify mix infection of trypanosome species (Desquesnes & Dávila 2002).

The ITS1 region has been successfully used to distinguish trypanosome species (Njiru et al. 2005). These authors documented specific PCR product length corresponding to each Trypanosoma species, which was the base of differentiation among Trypanosoma species. For example, *T. congolense savannah*, an ITS1 PCR product is 700 bp, 400 bp for *T. simiae* and 250 bp for *T. vivax.* The product for *T. evansi* and *T. brucei* subspecies was the same size, 480 bp. Herrera et al.



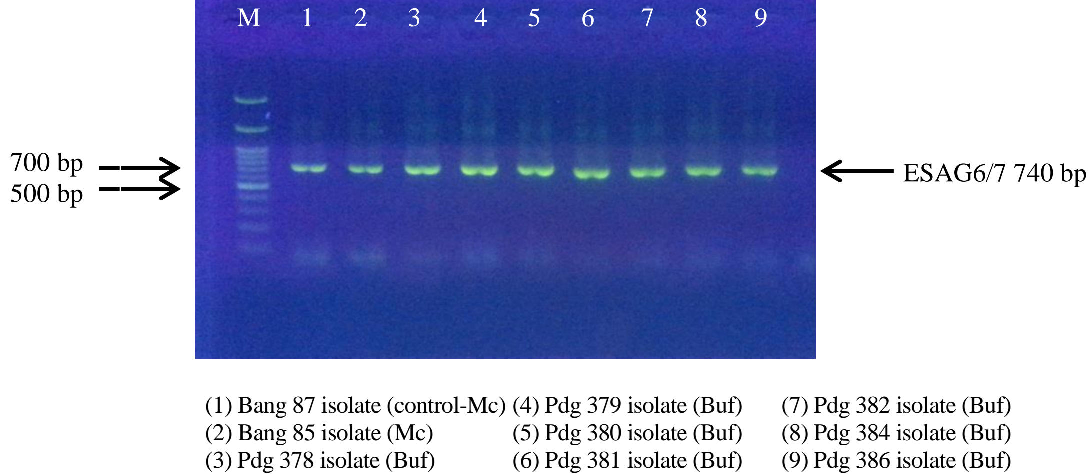

(1) Bang 87 isolate (control-Mc) (4) Pdg 379 isolate (Buf) (7) Pdg 382 isolate (Buf)
(2) Bang 85 isolate (Mc) (5) Pdg 380 isolate (Buf) (8) Pdg 384 isolate (Buf)
(3) Pdg 378 isolate (Buf) (6) Pdg 381 isolate (Buf) (9) Pdg 386 isolate (Buf)

**Figure 1**. PCR amplification of 480 bp ITS-1 gene of *T. evansi* from stabilates (Mc) and buffalo blood (Buf)




(1) Bang 87 isolate (control-Mc) (4) Pdg 379 isolate (Buf) (7) Pdg 382 isolate (Buf)
(2) Bang 85 isolate (Buf) (5) Pdg 380 isolate (Buf) (8) Pdg 384 isolate (Buf)
(3) Pdg 378 isolate (Buf) (6) Pdg 381 isolate (Buf) (9) Pdg 386 isolate (Buf)

**Figure 2**. PCR amplification of 151 bp Ro Tat 1,2 VSG gene of *T. evansi* from stabilates (Mc) and buffalo blood (Buf)




(1) Bang 87 isolate (control-Mc) (4) Pdg 379 isolate (Buf) (7) Pdg 382 isolate (Buf)
(2) Bang 85 isolate (Mc) (5) Pdg 380 isolate (Buf) (8) Pdg 384 isolate (Buf)
(3) Pdg 378 isolate (Buf) (6) Pdg 381 isolate (Buf) (9) Pdg 386 isolate (Buf)

**Figure 3**. PCR amplification of 740bp ESAG6/7 gene of *T. evansi* from stabilates (Mc) and buffalo blood (Buf)

(2001) reported that the highest sensitivity against primer was gold standard for *T. evansi*.

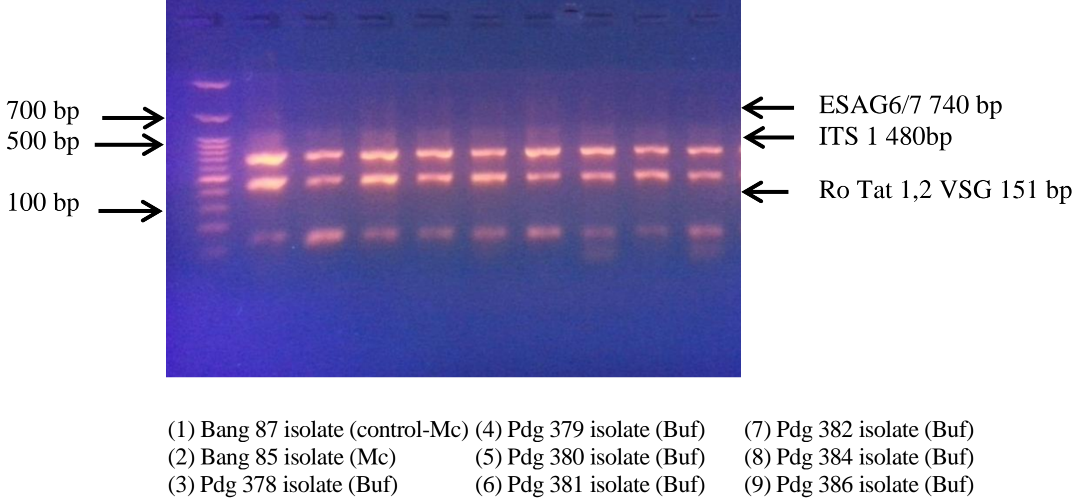
Specific PCR product for *T. evansi* by using Rotat 1.2 VSG gene was 151 bp (Konnai et al. 2009). Molecular marker using this gene was able to distinguish *T. evansi* strain type A (Ro Tat) and type B (non Ro Tat) (Njiru et al. 2006). Bajyana & Hamers (1988) successfully isolated protein RoTat 1.2 VSG from Indonesian *T. evansi* isolate which futher developed into diagnostic CATT 1.2 VSG kit. Ro Tat 1.2 VSG antigen was the predominant Variable Antigen Type (VAT) to be expressed during early, middle and late stages of infection (Verloo et al. 2001). Therefore, in this study, primer RoTat 1.2 VSG was picked as one of primers used to identified *T. evansi* from Indonesia. Njiru et al. (2006) and Claes et al. (2004) reported that *T. evansi* was divided into type A (RoTat 1,2 VSG) ciculating in Asia, Africa, Shouth America, and Middle America and type B (non RoTat 1.2 VSG) which circulating in Africa, especially in Kenya. Amplicon product in this study showed that *T. evansi* Indonesian isolate was type A and no one isolate that including the type B.

Another primer used in this study was ESAG 6/7, a gene located in VSG. PCR product of ESAG 6/7 *T. evansi* length was 740 bp and was able to be expressed by *T. evansi* type A and B (Isobe et al. 2003; Mekata et al. 2009). ESAG 6/7 was a sensitive and specific primer against trypanosome due to its multi-copy gene ability encodes *heterodimeric complex* on transferrin receptor (Pruvot et al. 2010; Kabiri & Steverding 2001). According to Schell et al. (1991) and Kabiri & Steverding (2001) *T. evansi* use transferrin receptor in the host's blood to obtain whole iron (Fe) serving in propagation phase. Transferrin receptor encoded by 2 expression-site-associated gen (ESAG6 and ESAG7) in VSG region. Difference ESAG sequence was reported able to cause different transferrin affinity towards different host (Bitter et al. 1998; Salmon et al. 1994; Steverding et al. 1995).

## Identification of *T. evansi* using multiplex PCR

Multiplex polymerase chain reaction (mPCR) is a variant of PCR in which two or more target loci from one or more organisms are amplified using a mixture of locus-specific primer pairs in a single reaction (Markoulatos et al. 2002). Result of multiplex PCR amplification on agarose gel 1.5% visualization under UV light showed three DNA fragment with specific size for *T. evansi*. PCR product by primer Ro Tat 1.2 VSG produce 151 bp fragment length. Besides, ITS-1, and ESAG6/7 fragment length were 480 bp and 740 bp respectively (Figure 4). Multiplex PCR amplification product was same size with the single one (Table 4). The results of DNA amplification samples derived from stabilate (BCC) and buffalo blood (*T. evansi* circulating isolates in 2012-2014) are the same. Therefore, *T. evansi* identification by multiplex PCR against ITS-1, RoTat 1.2 VSG and ESAG6/7 also showed that 31 trypanosome isolates were *T. evansi*.

This result showed that multiplex PCR analysis by mixing three primer pairs in one reaction successfully marked with three DNA fragment in every column in the gel. During this time, identification of trypanosome species including *T. evansi* was carried out by single PCR (Sukanto et al. 2000; Njiru et al. 2005; Njiru et al. 2004). Single PCR reaction for *T. evansi* detection and identification in large number of samples was expensive and time consuming (Ahmed et al. 2013). It was required two or more primer pairs to identify one isolate




(1) Bang 87 isolate (control-Mc) (4) Pdg 379 isolate (Buf) (7) Pdg 382 isolate (Buf)
(2) Bang 85 isolate (Mc) (5) Pdg 380 isolate (Buf) (8) Pdg 384 isolate (Buf)
(3) Pdg 378 isolate (Buf) (6) Pdg 381 isolate (Buf) (9) Pdg 386 isolate (Buf)

**Figure 4**. Amplification product of multiplex PCR against ITS-1 (480 bp), Ro Tat 1.2 VSG (151 bp) and ESAG6/7 (740 bp) gene of *T. evansi* from stabilates (Mc) and buffalo blood (Buf)

**Table 4**. *T. evansi* isolate sample obtained from BCC and Departement of Parasitology, BBlitvet, Bogor

| Isolate code | PCR product against *T. evansi* | | | |
|---|---|---|---|---|
| | Single | | | Multiplex |
| | ESAG6/7 | ITS-1 | Ro- Tat 1,2 VSG | |
| Bang 87 | + | + | + | + |
| Bang 85 | + | + | + | + |
| Pml 287 | + | + | + | + |
| Pml 291 | + | + | + | + |
| Sbw 340 | + | + | + | + |
| Sbw 341 | + | + | + | + |
| Sbw 363 | + | + | + | + |
| Sbw 364 | + | + | + | + |
| Sbw 366 | + | + | + | + |
| Smi 68 | + | + | + | + |
| Smi 369 | + | + | + | + |
| Bwi 218 | + | + | + | + |
| Bwi 222 | + | + | + | + |
| Bwi 228 | + | + | + | + |
| Ash129 | + | + | + | + |
| Ash 133 | + | + | + | + |
| Smb 370 | + | + | + | + |
| Smb 371 | + | + | + | + |
| Smb 372 | + | + | + | + |
| Smb 373 | + | + | + | + |
| Smb 374 | + | + | + | + |
| Smb 375 | + | + | + | + |
| Lbk 376 | + | + | + | + |
| Munt377 | + | + | + | + |
| Pdg 378 | + | + | + | + |
| Pdg 379 | + | + | + | + |
| Pdg 380 | + | + | + | + |
| Pdg 381 | + | + | + | + |
| Pdg 382 | + | + | + | + |
| Pdg 384 | + | + | + | + |
| Pdg 386 | + | + | + | + |
| Pdg 388 | + | + | + | + |

(Salim et al. 2011). Therefore, in this study multiplex PCR method was developed using more than 2 primer pairs in 1 PCR process. The multiplex PCR was cheaper because it only used one reaction in amplifying some fragment targets and needed a shorter time. This method was apllied in some diagnostic tests such as: detection of mixed *T. cruzi* and *T. rangeli* infection (de Sá et al. 2013), *T. evansi* and *Babesia bigemina* in India

(Sharma et al. 2012). This study was the first report that used multiplex PCR for *T. evansi* detection.

Using mPCR to make a diagnosis is three to five times cheaper than using the classical species-specific primers, as the number of reactions required per sample is reduced to a single one. Njiru et al. 2005 and Davila et al. 2003 stated the use of many primers can also lead to the identification of multiple infection of unexpected trypanosome species, especially in wild hosts, vectors and field stocks. This test might indentify targeted trypanosome species without cross amplification between the targeted genes of different trypanosome species. This technique ensure a permanent screening of any unexpected trypanosome species that could grow in vivo or in vitro as a mixed infection.

## CONCLUSION

Development of molecular detection technique of trypanosome DNA by mPCR using ITS-1, RoTat 1.2 VSG, and ESAG 6/7 primers has resulted in a considerable improvement of species-specificity in the diagnosis of these parasites to species level. mPCR success to amplify target gene from *T. evansi* sample from endemic and outbreak areas in Indonesia which was isolated since 1988-2014. Thirty one trypanosome isolates used in this study were *T. evansi* type A which circulating in Asia. This technique recommended to be used in field.

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Microencapsulation of bull spermatozoa: Its viability in alginate-egg yolk media (Mikroenkapsulasi spermatozoa sapi: Daya hidup spermatozoa pada media alginat-kuning telur)
(Org: Ind)

JITV 20(1): 1-9

Microencapsulation of spermatozoa is a process to entrap a number of spermatozoa in microcapsule. Alginate, as a natural polymer polysaccharide is commonly used in cell microencapsulation. Tris Yolk Citrate buffer is a good buffer for spermatozoa dilution, therefore this experiment aimed to determine optimal concentration of alginate and egg yolk to sperm quality in bull spermatozoa microencapsulation. Concentration of egg yolk and alginate in media of encapsulation were determined in applications of sperm microencapsulation. Four bulls were used as semen source and only semen with good quality were used in this study. Poolled semen was diluted using the medium to get final concentration 100 x $10^6$ cell/ml. The first study was conducted to determine the effect of concentration of alginate (0, 1, and 1.5%) on viability of spermatozoa. The second study to determine the effect of alginate concentration, egg yolk and its interaction was done by comparing two levels of alginate (1 and 1.5%) with four levels of egg yolk (5, 10, 15 and 20%). Viability of spermatozoa, motility (M), live spermatozoa (L) and Intact Apical Ridge (IAR) were observed at 0, 1, 2 and 3 h incubation at room temperature. Results indicated that alginate concentration increased the osmolality and viscosity but did not affect pH of the medium. The osmolality and viscosity of medium were 275, 325, 425 and 1.12, 26.62, 47.98 for concentration of alginate 0, 1 and 1.5% respectively. Percentage of motility is significantly lower ($P<0.05$) in alginate medium than those of control, and 1.5% alginate could produce more uniform beads. Concentration of alginate, egg yolk and its interaction did not significantly affect viability of sperm. It is concluded that the combination of 1.5% alginate with 5, 10, 15 or 20% egg yolk can be used as media for sperm encapsulation.
(Author)
**Key Words**: Microencapsulation, Spermatozoa, Sodium Alginate, Egg Yolk, Viability

**UDC: 636.2.034**

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Analisis faktor-faktor genetik dan non genetik sifat-sifat reproduksi dan produksi susu sapi Friesian-Holstein di Mesir (Genetic and non-genetic analysis for milk production and reproductive traits in Holstein cattle in Egypt)
(Org: Eng)

JITV 20(1): 10-17

This study was carried out to investigate genetic, non-genetic affecting factors and estimate genetic parameters for milk production and reproductive traits of Holstein cows via animal model. The 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. The means and coefficient of variability (CV%) of milk traits as total milk yield (TMY), 305 days milk yield (305-dMY), lactation period (LP) and dry period (DP) were 5787.8 kg (31.1%), 4695 kg (22.1%), 332 days (14.9%) and 72.3 days (27.7%), respectively. Also, the means (CV%) of reproductive traits as days open (DO) and age at first calving (AFC) were 157.9 days (22.6%) and 30.5 month (16.8%), respectively. Sire, dam, parity of cow, year and season of calving had significant effects on traits studied. Heritability estimated were 0.223, 0.184, 0.112, 0.118, 0.105 and 0.285 for TMY, 305-dMY, LP, DP, DO and AFC, respectively. Estimated $r_G$ and $r_P$ among milk production traits were positive but it takes negative trend with DP and DO. Moderate heritability estimates and positive genetic correlation for most of traits studied suggested that genetic improvement of these traits would be achieved via multi-trait selection.
(Author)
**Key Words**: Genetic Parameters, Milk Production, Reproductive, Friesian Holstein Cattle

**UDC: 636.597**

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Karakteristik sifat rontok bulu dari persilangan itik Alabio dan Peking (Molting characteristics of crossbreds between Alabio and Pekin ducks)
(Org: Eng)

JITV 20(1): 18-22

Molting is a problem for duck farmers, during which the ducks stop laying eggs. Molting characteristics may be altered through crossing between non-molting to molting ducks. The purpose of this study was to evaluate molting characteristics of the crossing between Alabio and Pekin ducks. The materials used were 90 female AP (Alabio males x Pekin females) and 90 female PA (Pekin males x Alabio females), 25 female Alabio and 25 female Pekin ducks. The ducks were housed in individual cages at the Indonesian Research Institute for Animal Production, Ciawi Bogor. Variables measured were the length of molting and egg production in 30 weeks, data were analyzed using ANOVA and followed by the estimation of the value of heterosis. Result showed that the molting of Pekin ducks was 71.31±9.36 days, it was longer than Alabio, AP or PA ducks, which were respectively 42.44±8.59, 43.63±4.88 and 49.35±4.85 days (P<0.01). Egg production of Pekin duck (56.41±4.59) was significantly lower (P<0.01) than that of Alabio, AP or PA, which were 72.48±3.24, 83.75±1.39 and 76.12±1.68 respectively. The heterosis of molting period of AP was higher than that of PA (-23.29 vs -13.23%). The heterosis of egg production of AP was higher than PA (29.96 vs 18.12%). It is concluded that the AP crossbred could be utilized as the initial population to produce a superior line characterized by high egg production and controllable molting.

(Author)

**Key Words:** Duck, Alabio, Pekin, Crossbreds, Molting

**UDC: 636.32/.38**

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Kecernaan nutrisi dan pertumbuhan lima rumpun domba pada beberapa tingkat kandungan protein tidak terdegradasi (Nutrient digestibility and growth of five breeds of sheep under different levels of undegradable protein)

(Org: Ind)

JITV 20(1): 23-30

A study was conducted to evaluate the effect of feeding different levels of rumen undegradable protein (UDP) in concentrate on performance of five breeds of sheep. Namely: Compass Agrinak (CA); Garut Composite breed (KG); Barbados Cross breed (BC); St Croix breed (SC); and Local Garut (GL) breed. Ten heads of sheep were used from each breed, in which each breed was grouped into 5 groups according to their body weight, and each sheep in each group was assigned to one of two treatments diet. The diet treatment consisted of concentrate containing different levels of undegradable protein (UDP). The levels of undegradable protein were 4.5% and 7.5%. Concentrate were formulated in iso nitrogen (CP content 16.7%) and iso energy (ME content 2.5 Mcal/kg). During feeding trial the sheep were kept in individual pen for three months. Study was conducted in randomized complete block design in factorial 5 x 2 arrangement, 5 levels of breeds sheep and 2 levels of UDP content. Results shows that there was no interaction effect of breed and levels UDP on feed consumption, average daily gain and feed conversion. These variables were significantly (P<0.05) affected by breed of sheep. The highest DMI (dry matter intake) was in KG sheep, the highest DMI caused by highest crude protein and gross energy intake, which in turn lead to highest average daily gain (ADG) of this breed. However the ADG of KG was not significantly different from GL. Apparent nutrient digestibility was not affected (P>0.05) by interaction between breed of sheep and UDP levels in the diet except for OM digestibility. While breed of sheep did not affect nutrient digestibility and UDP levels only affected NDF digestibility. From this study, it is concluded that increasing UDP in the diet did not improve growth performance of sheep. At similar quality of feed the growth performance was affected by breed of sheep in which KG and GL sheep had similar average daily gain (86.01 vs 82.38 g/day).

(Author)

**Key Words:** Breed of Sheep, Undegradable Protein, Growth, Digestibility

**UDC: 636.085.52**

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Digestion and ruminal fermentation of cocoa pod silage based ration enriched by gliricidia and calliandra leaves on goats (Kecernaan dan fermentasi ruminal ransum berbasis silase kulit buah kakao yang diperkaya daun gamal dan kaliandra pada kambing*)*

(Org: Ind)

JITV 20(1): 31-40

In term of availability, cacao pod is potential for ruminant feed. According to its nutrients content, cacao pod can be used as feed fiber source. Protein sources materials must be added when cacao pod was ensilaged due to low protein content of this material. The aim of this study was to investigate digestibility value and end products of rumen fermentation of goat fed grass or cacao pod based ration. Randomized block design and 20 heads of lambs (16.95±2.36 kg) to evaluated 5 type of rations: R (50% grass + 50% concentrate); S (50% cacao pod silage + 50% concentrate); SG (50% cacao pod-gliricidia silage + 50% concentrate); SK (50% cacao pod-calliandra silage + 50% concentrate) dan SC (50% cacao pod-mixture of gliricidia-calliandra silage + 50% concentrate). Feeding trial was conducted for over 15 weeks. Measurements were taken on feed digestibility and rumen-fermentation end-products after 3 weeks of treatments. Results shows that nutrients digestibility was different significantly among the groups of treatments (P<0.05). Digestibillity of organic matter, NDF and energy of R ration was those of higher significantly (P<0.05) than those of other groups. N-ammonia of rumen from goat feed R ration was higher (P<0.05) than other groups. Total VFA and each component were different among the groups (P<0.05), however the value was similar among the groups of cacao pod silage rations. It is concluded that cacao pod silaged based rations enriched by Gliricidia and Calliandra leaves did

not produce similar digestibility value and end products of rumen fermentation with grass based ration.

(Author)

**Key Words**: Cacao Pod Silage, Digestibility, Ruminal Fermentation

**UDC: 636.2.033**

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Mayberry, D. (CSIRO Ecosystem Sciences, Dutton Park, Queensland, Australia)
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Penambahan konsentrat pada ransum sapi jantan persilangan untuk meningkatkan keuntungan peternak sapi penggemukkan di Jawa Timur, Indonesia (Concentrate supplementation for crossbred bulls to increase profitability of smallholder fattening operations in East Java, Indonesia*)*

(Org: Eng)

JITV 20(1): 41-47

Growth rates of bulls in Indonesia raised in smallholder fattening systems are generally low due to inadequate nutrition. This study compared bull growth and farmer profit between two feeding management systems, namely traditional feeding (as control) and intervention feeding by supplementation with a high quality concentrate. Bos taurus-Ongole cross bred bulls (n=46) aged 1.5-2 years, owned by smallholder farmers, from two villages in East Java were used in this study. The bulls were divided into two treatment groups; control and intervention. Farmers in the intervention group were provided with a concentrate containing onggok, copra and palm kernel cake. Farmers were instructed to feed 4 kg DM of concentrate/bull/day in addition to their existing roughage diet for 161 days or until sold. No changes were made to the feeding or management of the bulls in the control group. Mostly control group farmers used local forages and agricultural by product as a feed, without any supplementation. The daily feed cost and the cost of 1 kg of liveweight gain was higher in the intervention group (IDR 8827 and IDR 11990, respectively) than that of the control group (IDR 2606 and IDR 5543, respectively). Moreover, the average daily gain of bulls in the intervention group (0.82 kg/bull/day) was higher than that of bulls in the control group (0.52 kg/bull/day), resulting in a greater profit for the fattening period. Daily income minus feed costs was higher for farmers in the intervention group (IDR 24182/day), compared to farmers in the control group (IDR 15774/day). It is concluded that although there were additional costs for feeding high-quality feeds, but higher growth rates of bulls resulted in greater profits for smallholder farmers.

(Author)

**Key Words**: Feed, Fattening, Beef Cattle, Supplement, Concentrate

**UDC: 636.39**

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Effect of protein levels and Zinc-biocomplex supplementation in concentrate diets on performance of young male goats (Pengaruh tingkat protein dan penambahan Zn biokompleks dalam konsentrat terhadap performa kambing jantan muda)

(Org: Ind)

JITV 20(1): 48-57

This trial was carried out to investigate effects of protein levels and Zinc biocomplex supplementation in concentrate diets on performances of young male Etawah grade goats. Twenty-four young male goats were divided into four groups and received concentrate diets as follows: $R_0$= 14% crude protein (CP), $R_1$= 18% CP, $R_2$= $R_0$ + 60 ppm Zn and $R_3$= $R_0$ + 120 ppm Zn as Zn biocomplex. Initial live weight was 16.39±2.19 kg. Animals were offered King grass ad libitum and 400 g/h/d of concentrates diets for 16 week trial. The experiment was conducted based on a randomized complete design with four treatments and six replications. The concentrate diets had no significant effect on DM, TDN, NDF and ADF daily intakes (P>0.05) but significantly (P>0.05) influenced the CP and Zn daily intakes, ADG and FCR. The average DMI, TDN, NDF and ADF daily intakes for all treatments were 670, 547, 333 and 229 g, respectively. The CP daily intake for $R_0$, $R_1$, $R_2$ and $R_3$ treatments were 76.33, 91.83, 75.83 and 76.67 g, and the Zn daily intakes were 42.83, 45.50, 68.83 and 91.33 mg, respectively. The ADG for $R_0$, $R_1$, $R_2$ and $R_3$ were 71.65, 79.96, 78.17 and 82.74 g with the FCR values were 9.95, 8.50, 8.44 and 8.06, respectively. The in vivo digestibility of DM, NDF and ADF were not significant (P>0.05) but the digestibility of CP and GE were significant (P<0.05). The highest IOFC value occurred at $R_3$ treatment. In conclusion, the improvement of CP levels from 14% to 18% in diets increased the goat performance and the supplementation of 120 ppm Zn as Zn biocomplex in diet containing 14% CP gave better performance and increased the IOFC value compared to animals receiving 18% level of CP in diet of young male goat.

(Author)

**Key Words:** Goats, Concentrates, Performances, Protein, Zn Biocomplex

**UDC: 636.597**

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Performance of EPMp broiler duck fed various levels of lysine during starter period (Performa itik pedaging EPMp

dengan pemberian pakan yang mengandung berbagai level lisin selama periode starter)

(Org: Ind)

JITV 20(1): 58-63

The aim of this study was to determine optimal requirement of lysine of broiler EPMp ducks during starter period. The study was designed in a completely randomized design (CRD) with four dietary treatments, four replications, and each replication consisted of 10 ducks. The treatments were: T1 (ration, with 0.70% digestible lysine); T2 (ration, with 0.85% digestible lysine); T3 (ration, with 1.00% digestible lysine); T4 (ration, with 1.15% digestible lysine). Variables measured were: feed intake, body weight gain and feed conversion ratio (FCR). Results showed that average body weight gain of EPMp broiler ducks was significantly affected ($P<0.05$) by the level of lysine in the diet, but feed intake and FCR were not significantly ($P>0.05$) affected. Mean body weight gain of EPMp broiler duck with T4 ration (1.15%) of lysine was significantly higher compared to T3 ration (1.00% of lysine), but between T4 to T1 and T2 treatmeants were not significantly different ($P>0.05$). T3 treatment compared to T1 and T2 treatments were not significantly different ($P>0.05$). There is a pattern of decreasing feed consumption and FCR by increasing content of lysine in the diet, protein and lysine consumption during the starter period. It is concluded that administration of digested lysine at 0.70 and 0.85%, protein and metabolized energy respectively by 18% and 2800 kcal/kg EM in feed were considered sufficient to generate performance (feed consumption, body weight gain and FCR) of EPMp broiler ducks in starter period.

(Author)

**Key Words:** Performance, EPMp Ducks, Lysine, Starter

**UDC: 578.76**

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Protection level of AI H5N1 vaccine clade 2.1.3 commercial against AI H5N1 clade 2.3.2 virus from Ducks to SPF chicken in laboratory conditions (Tingkat perlindungan vaksin komersial AI H5N1 clade 2.1.3 terhadap virus AI H5N1 clade 2.3.2 asal itik pada ayam SPF dalam kondisi laboratorium)

(Org: Ind)

JITV 20(1): 64-70

Highly Pathogenic Avian Influenza (HPAI) subtype H5N1 clade 2.3.2 has infected chickens in farms, causing mortality and a decrease in egg production. Vaccination is one of the strategies to control disease of AI subtype H5N1. AI H5N1 clade 2.1.3 vaccine is available commercially. The effectiveness of two vaccines of AI H5N1 clade 2.1.3 (product A and B), and AI H5N1 clade 2.3.2 (Sukoharjo) against AI H5N1 clade 2.3.2 (Sukoharjo) virus SPF chickens was tested in laboratory. Four groups of SPF chickens were used in this study, there were (1) vaccinated with H5N1 clade 2.1.3 (product A), (2) vaccinated with H5N1 clade 2.1.3 (product B), (3) vaccinated with AI H5N1 clade 2.3.2 and (4) unvaccinated (as a control). Each vaccinated group consisted of 10 chicken except 8 chicken for control group. SPF chicken were vaccinated with 1 dose of vaccine at 3 weeks olds, and then after 3 weeks post vaccination (at 6 weeks olds). All group of chicken were challenged with $10^6$ $EID_{50}$ per 0.1 ml via intranasal. The results showed, chicken vaccinated with H5N1 clade 2.1.3 product A and B gave 100 and 80% protection respectively, but showed challenged virus shedding, whereas vaccine of H5N1 clade 2.3.2 gave 100% protection from mortality and without virus shedding. Vaccines of AI H5N1 clade 2.1.3 product A was better than vaccine product B, and when chicken vaccinated against H5N1 clade 2.3.2, H5N1 clade 2.3.2 vaccine was the best to be used. In order to protect chicken from AI subtype H5N1 clade 2.1.3 and 2.3.2 in the field, a bivalent vaccine of H5N1 clade 2.1.3 and 2.3.2 subtypes should be developed.

(Author)

**Key Words:** Chicken, HPAI, Local, Vaccine

**UDC: 632.3**

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Effectivity of PCR and AGID methods to detect of enzootic bovine leukosis in Indonesia (Efektivitas metode PCR dan AGID dalam mendeteksi penyakit Enzootic Bovine Leucosis di Indonesia)

(Org: Ind)

JITV 20(1): 71-78

Enzootic Bovine Leucosis (EBL) is one of viral diseases in cattle caused by bovine leukemia virus (BLV), from Retroviridae. The virus can be detected using severals methods such as Polymerase Chain Reaction (PCR), while antibody can be detected using Agar Gel Immunodifussion (AGID). The aim of this experiment was to study the effectivity of PCR and AGID methods to detect enzootic bovine leukosis virus in Indonesia. Samples of peripheral blood leukocyte (PBL) were collected from cattles those with and without showing clinical signs. A total of 307 blood and serum samples were tested against BLV using PCR and AGID tests, while 21 semen samples which were from similar animals for blood collection were collected only for PCR test. The results indicated that twelve cattles have positive results with PCR test in PBL, but from those cattles only seven were positive with AGID. On the other hand, the PCR did not detect EBL in 21 bovine semen samples tested, although one sample gave positive result with PCR in PBL. This results indicated that PCR method from blood samples was more sensitive than that AGID method. The PCR detection was also more sensitive for PBL than that for semen samples.

(Author)

**Key Words:** Enzootic Bovine Leucosis, Indonesia

**UDC: 636.2**

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Analisis *multivariate* sifat-sifat morfometrik pada tiga populasi sapi asli bagian Timur Laut India yang berbeda (Multivariate analysis of morphometric traits of three different indigenous cattle populations from North East states of India)

(Org: Eng)

JITV 20(2): 79-86

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

(Author)

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

**UDC: 636.293.2**

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Isolasi dan identifikasi bakteri asam laktat indigenus susu kerbau sungai Sumatera Utara (Isolation and identification of indigenous lactic acid bacteria from North Sumatra river buffalo milk)

(Org: Eng)

JITV 20(2): 87-94

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

(Author)

**Key Words:** Buffalo Milk, LAB, Isolation, Identification

**UDC: 636.3**

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Efektivitas probiotik, mikromineral terkorporasi khamir yang dikombinasikan dengan daun *Azadirachta indica* mengandung tanin terhadap fermentabilitas dan kecernaan *Pennisetum* hybrid (Effectivity of probiotic, micromineral enriched yeast and their combination with *Azadirachta indica* leaves containing tannin on fermentability and digestibility of *Pennisetum* hybrid)

(Org: Eng)

JITV 20(2): 95-104

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

(Author)

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

**UDC: 633.3**

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Analisis produktivitas tanaman *Caliandra calothyrsus*, *Indigofera zollingeriana* dan *Gliricidia sepium* pada lahan kering masam di rumah kaca (Productivity *of Calliandra calothyrsus, Indigofera zollingeriana* and *Gliricidia sepium* on acid soil in the greenhouse)

(Org: Eng)

JITV 20(2): 105-114

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

(Author)

**Key Words:** Leguminosa Pohon, Tanah Masam, $Al^{3+}$

**UDC: 578.2**

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Analisis molekuler gen penyandi hemaglutinin virus Avian Influenza isolat 2012-2013 (Molecular analysis of hemaglutinin gene of Avian Influenza viruses isolated in 2012-2013)

(Org: Eng)

JITV 20(2): 115-125

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

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

**UDC: 303.214.2**

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Reliabilitas tes DIVA berbasis M2e jauh lebih tinggi dibandingkan dengan tes DIVA berbasis HA2 atau protein NS1 (The reliability of DIVA test based on M2e peptide exceed those based on HA2 or NS1 peptides)

(Org: Eng)

JITV 20(2): 126-133

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

(Author)

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

**UDC: 578.24**

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Kajian infeksi virus Newcastle Disease pada bebek dan ayam di Kabupaten Subang (Newcastle Disease Virus infection study on duck and chicken in Subang district)

(Org: Eng)

JITV 20(2): 134-147

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

with relatively low antigenic variation among circulating strains.

(Author)

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

**UDC: 619:616.24**

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Pengaruh kurkumin terhadap fibrosis paru-paru akibat aplikasi bleomisin pada *Mus musculus* (Curcumin effect on Bleomycin-induced pulmonary fibrosis in *Mus musculus*)

(Org: Eng)

JITV 20(2): 148-157

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

(Author)

**Key Words**: Bleomycin, Curcumin, Mice, Pulmonary Fibrosis

**UDC: 636.2.034**

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Indeks seleksi multi-trait dan multi-source untuk sifat-sifat produksi susu dan reproduksi pada sapi Holstein di Mesir (Multi-trait and multi-source selection indices for milk production and reproductive traits in a herd of Holstein cattle in Egypt)

(Org: Eng)

JITV 20(3): 159-167

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.

(Author)

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

**UDC: 636.2.033**

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Estimasi nilai heritabilitas dan faktor non genetik yang mempengaruhi sifat-sifat produksi sapi PO (Heritability estimation and non genetic factors affecting production traits of Indonesian Ongole cross)

(Org: Eng)

JITV 20(3): 168-174

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 $0.28\pm0.12$, $0.47\pm0.15$ and $0.63\pm0.17$ 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.

(Author)

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

**UDC: 613.287.6**

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Aktivitas antimikroba dan antioksidan peptida hasil hidrolisis susu kambing dengan berbagai protease (Antimicrobial and antioxidative activities of peptides from goat milk hydrolyzed with various protease)

(Org: Eng)

JITV 20(3): 175-183

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.

(Author)

**Key Words**: Goat Milk, Peptide, Antimicrobe, Antioxidant

**UDC**: **636.58.033**

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Pengaruh penambahan enzim terhadap nilai gizi bungkil inti sawit terfermentasi untuk menggantikan bungkil kedelai dalam ransum ayam broiler (Effect of enzyme supplementation on nutritive values of fermented palm kernel cake used to substitute soybean meal in broiler diet)

(Org: Eng)

JITV 20(3): 184-192

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.

(Author)

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

**UDC: 637.4'658**

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Pemberian ekstrak *Salix tertrasperma* kombinasi dengan ekstrak kunyit dan mimba untuk peningkatan kualitas telur ayam yang dipelihara pada suasana stres panas (Administration of extract *Salix tetrasperma* combined with extract of turmeric and neem to improve eggs quality of chicken reared under heat stress)

(Org: Eng)

JITV 20(3): 193-199

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.

(Author)

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

**UDC: 637.54'659.7**

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Profil lipid darah dan komposisi asam lemak daging itik hibrida dengan suplementasi tepung buah Mengkudu (*Morinda citrifolia*) dalam pakan (Lipid profiles of blood serum and fatty acid composition of meat of hybrid duck fed diet supplemented with Noni (*Morinda citrifolia*) fruit meal)

(Org: Eng)

JITV 20(3): 200-206

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.

(Author)

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

**UDC: 582.736**

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Produksi dan kualitas biomassa *Murdannia bracteata* sebagai dampak aplikasi pupuk daun magnesium (Production and quality of *Murdannia bracteata* biomass as impact of magnesium foliar fertilizer)

(Org: Eng)

JITV 20(3): 207-213

*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.

(Author)

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

**UDC: 591.2**

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Perubahan patologi pada sapi potong yang diduga terpapar tetrachloro dibenzo-ρ-dioxins/tetrachloro dibenzofurans (Pathological changes of suspected tetrachloro dibenzo-ρ-dioxins/tetrachloro dibenzofurans toxication in beef cattle)

(Org: Eng)

JITV 20(3) 214-223

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.

(Author)

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

**UDC: 632.3**

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Peredaran virus H5N1 pada ayam buras di sekitar peternakan ayam petelur komersial (Circulating H5N1 virus among native chicken living around commercial layer farms)

(Org: Eng)

JITV 20(3): 224-232

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.

(Author)

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

**UDC: 581.35**

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Mikroenkapsulasi spermatozoa sapi: Kriopreservasi mikroenkapsulasi spermatozoa menggunakan gliserol (Microencapsulation of bovine spermatozoa: Cryopreservation microencapsulation of sperm using glycerol)

(Org: Eng)

JITV 20(4): 233-241

Cryopreservation of spermatozoa has been used to preserve spermatozoa in very low temperatures. Glycerol is intracelullar cryoprotectant usually used in Tris citrate containing 20% egg yolk (TCEY), while TCEY-1.5% alginate was used as encapsulation media. The effect of alginate in microencapsulation process and glycerol concentration on viability spermatozoa (motility (%M), live sperm (%L) and intact apical ridge (%IAR) were studied in two steps. In the first step, the effect of alginate and microencapsulation process was studied. Three treatments of this step were: 1) TCEY add with semen (as control), 2) TCEY-Alginate add with semen, 3) TCEY-Alginate add with semen and microencapsulated, followed by conventional sperm cryopreservation using TCEY 7% glicerol. The second steps were done to evaluate the effect of two glycerol concentrations (5 and 7%) and two duration of equilibration time (3 and 4 hours). Result of these experiments showed, that microencapsulation was significantly alter the percentage of post-thawing motility (%M) and intact apical ridge (%IAR). The motility of microencapsulated sperm vs control was 44.7 and 50.83% respectively, whereas IAR 79.33% and 83.50% on microencapsulated sperm and control. There was evidence that alginate act as extracelullar cryoprotectant by protecting sperm during freezing. The mean of decreasing percentage of %M and %IAR in TCEY-Alginate were lower (15.97 and 6.44%) than control (23.80 and 7.37%). The effect of glycerol concentration and equilibration time on the viability of sperm was not significant different. There was no significant interaction of glicerol and equilibration time on the viability of spermatozoa. In conclution, microencapsulation processes altered the viability of sperm, alginate had an important roles as extracelullar cryoprotectant. Moreover the cryopreservation of microencapsulated sperm might be done using 5 or 7% glycerol in 3-4 hours duration of equilibration time.

(Author)

**Key Words:** Sperm, Alginate, Microencapsulation, Glycerol, Equlibration Time

**UDC: 636.293.2**

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Karakteristik produksi gas dan fermentasi rumen pada ransum kerbau yang mengandung hasil samping dari beberapa varietas tanaman sorgum (Gas production and rumen fermentation characteristics of buffalo diets containing by-product from some sorghum varieties)

(Org: Eng)

JITV 20(4): 242-249

Sorghum is one of potencial fibre sources as buffalo feed. Quality of sorghum could be increased by irradiation mutation breeding. Samurai 1 and Samurai 2 were products of the irradiation mutation breeding of Pahat. This study was conducted to compare buffalo diets containing Samurai 2 sorghum straw and Samurai 1 bagasse sorghum compared with Pahat sorghum straw using in vitro study. Completely randomized design with 6 treatments and 3 replications was applied in this experiment. The treatment diets were P1 (50% Pahat sorghum straw + 50% concentrate), P2 (50% Pahat sorghum straw silage + 50% concentrate), P3 (50% Samurai 2 sorghum straw + 50% concentrate), P4 (50% Samurai 2 sorghum straw silage + 50% concentrate), P5 (50% Samurai 1 sorghum bagasse + 50% concentrate) and P6 (50% Samurai 1 sorghum bagasse silage + 50% concentrate). The 200 mg DM samples of diets were incubated in 30 ml rumen-buffer fluid for 48 hours. Variables measured were total gas production, CH4 production and rumen fermentation characteristics. Results showed that P2 and P4 produce the highest of gas production ($P<0.05$) with 60.99 and 60.86 ml/200 mg dry matter respectively. Treatments of P1, P2 and P4 produced the lowest CH4 concentration ($P<0.05$) with 10.57, 10.90 and 9.82% of total gas, respectively. The P4 produced the highest total volatile fatty acids (VFA), dry matter degradability and organic matter degradability with 109.83 mM, 62.93% and 59.97% respectively, meanwhile ammonia (NH3) concentration was not significantly different. The conclusion showed that straw silage of Samurai 2 was comparable to the Pahat sorghum straw and Samurai 1 bagasse sorghum as buffalo diet.

(Author)

**Key Words**: Buffalo, CH4 Concentration, Rumen Fermentation Characteristics, Sorghum

**UDC: 636.38**

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Peran limbah jeruk manis (*Citrus sinensis*) dalam menurunkan kadar kolesterol dan lemak daging pada domba Padjadjaran (Role of sweet orange (*Citrus sinensis*) waste in lowering the meat cholesterol and fat of Padjadjaran sheep)

(Org: Eng)

JITV 20(4): 250-256

This research is aimed to evaluate sweet orange's role in lowering meat cholesterol and fat in Padjadjaran sheep. Twenty sheeps with body weigth 29.66±2.74 kg and variance coefficient <10% were used in this research using Completely Randomize Design (CRD). The treatments were consist of four level sweet orange addition in ration. The treatments were T1 (0%), T2 (7%), T3 (12%) and T4 (17%) that were repeated 5 times. The research was conducted for five weeks with observed variables are ration consumption that was measured everyday, body gain that was measured every week, cholesterol and triglycerides measured at the end of the research. During the research water consumption was given ad libitum. Meat samples were selected randomly from three for each repeated treatments. Observation result showed the highest decrease on meat cholesterol level was T2 treatment with 9.43±0.04 mg/mg on thigh and 9.71±0.04 mg/mg on breast. Meanwhile for fat, the highest decrease was from T4 treatment with 9.70±3.98 ug/mg dan 10.48±1.85 ug/mg. Rumen's pH did not show significant difference (P>0.05) on all treatments with value range between 6.80±0.01 – 6.5±0.02. In conclusion, sweet orange (*Citrus sinensis*) waste is capable to lowering cholesterol level and fat in meat of Padjadjaran sheep.

(Author)

**Key Words:** Sweet Orange Waste, Meat Cholesterol, Meat Fat, Padjadjaran Sheep

**UDC: 636.38**

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Karakteristik suara beberapa rumpun domba dan pemanfaatannya untuk pendugaan jarak genetik (Voice characteristics of some sheep: Utilization to estimation of genetic distance)

(Org: Eng)

JITV 20(4): 257-267

Sound analysis has been carried out in various activities including identification and differentiation of species as well as the preparation of the taxonomy of some animals' species because of several advantages, including no need to capture or too close to the subject observed. Analysis of voice used to differentiate and to estimate of breeds' sheep genetic distance has not been reported. This research was conducted to study the character of a few breeds' sheep sound and likely to be used as a predictor of genetic distance between breeds of sheep. The study was conducted in the Animal House at Indonesian Research Institute for Animal Production, Bogor. A total of 20 head adult of five sheeps (St. Croix cross/SC, Barbados Black Belly cross/BC, Local Garut/LG, Composite Garut/KG and Composites Sumatra/KS) used in this study. Call sound recorded using a digital voice recorder. Sound analysis performed by Raven Software Pro 1.3 for Windows to count as many as 24 variables sound. Analysis of variance of each variable sound was performed using PROC GLM of SAS software Ver. 9.0. It used PROC CANDISC for canonical discriminant analysis and then PROC TREE to build a dendogram. The results showed that there were variations in amplitude, energy, power and frequency variables among the five breeds of sheep. By plotting canonical, LG, KS and BC sheep were from a different group. It was concluded that the sound characteristics variables which can be used as a differentiator breeds of sheep were the third quartile frequency, center frequency, maximum frequency and the first quartile time. Dendogram showed that KG sheep was in the less accurate group. Genetic distance estimation method using voice characteristic data may be applied on sheep.

(Author)

**Key Words:** Characteristics, Call Voice, Differentiation, Genetic Distance, Sheep

**UDC: 636.085.2**

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Kecernaan karbohidrat dan protein pada bungkil inti sawit dengan enzim Mannanase BS4 dan papain koktil (Carbohydrate and protein digestion of palm kernal cake by Mannanase BS4 and papain cocktail enzymes)

(Org: Eng)

JITV 20(4): 268-274

Supplementation of the mannanase BS4 enzyme on palm kernel cake (PKC) increased its metabolisable energy (ME), and supplementation with protease is expected to increase its protein digestibility. Therefore, the purpose of this research is to determine the optimum proportion of cocktail enzymes between BS4 β-mannanase (produced by *Eupenicillium javanicum*) and protease (papain) and their degradation activities on carbohydrate and protein of PKC. The β-Mannanase was produced by the mold through solid substrate fermentation (SSF) using coconut meal as the substrate. The papain was extracted from papaya latex (PL), collected by longitudinal incisions on unripe papaya fruit and oven dried overnight. The evaluation of enzyme cocktails for PKC hydrolysis was done at pH 5.8 and 40°C which are similar with poultry intestine condition and both enzymes are still active. The β- mannanase BS4 : papain were mixed with some proportions, i.e.: 100 : 0; 75 : 25; 50 : 50; 25 : 75 and 0 : 100% (by volume) in order to study the optimum cocktail composition ratio. The activities of β-mannanase towards gum locust bean was 86 $U.ml^{-1}$ and papains PL activity was

18,000 U.g$^{-1}$respectively. PL was chosen for synergistic reaction and compared with a commercial Merck papain (CMP, 20,000 U.g$^{-1}$) as positive control. β-Mannanase BS4 showed carbohydrate digestion activity, and protein digestion activity was not detected. Papain showed protein digestion activity and no carbohydrate digestion activity. Enzyme cocktails of 50 : 50 from PL protease showed slightly increased in synergistic protein digestion activity in PKC. However, its reduction sugar production was much lower than 100 : 0 and 75 : 25. Amino acids production by enzyme cocktails 75 : 25 were higher than that of 100 : 0. As a result, the best volume composition of β-mannanase BS4 and papaya latex was 75 : 25 (v:v) or 14 : 10 (U:U).

(Author)

**Key Words**: Mannanase BS4, Papain, Cocktail-Enzymes, Palm Kernel Cake

**UDC: 615.371**

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Efektivitas trypanosidal terhadap beberapa isolat *Trypanosoma evansi* yang diperbanyak di mencit (Trypanocidals effectivity against some isolates of *Trypanosoma evansi* propagated in mice)

(Org: Eng)

JITV 20(4): 275-284

Surra is one of infectious diseases in various types of animals caused by blood parasites called *Trypanosoma evansi*. It is fatally occurred, especially in horse, buffalo and camel. Surra may be controlled by effectively trypanocidals treatment based on the results of its sensitivity test. Therefore, it is necessary to study the effectiveness of various trypanocidal against some *T.evansi* isolates originating from several regions in Indonesia with surra case to determine its suitability and efficacy. The test was carried out by pre-test - post-test. Mice were infected by several *T.evansi* isolates from various infected areas. Their parasitaemia were observed. After reaching peak of parasitemia, the mice were treated by trypanocidal with different doses. Parasitaemia alteration was observed for one month. Observation results showed that all isolates had different sensitivity to the trypanocidals, so that trypanocidals application tended to specific location. Generally, suramin and melarsomine dihydrochloride were the most effective trypanocidal for some Indonesian isolates. In contrast, isometamidium chloride was not recommended to be used for surra control in Indonesia.

(Author)

**Key Words:** *Trypanosoma evansi*, Trypanocidal, Parasitemia, Surra

**UDC: 632.38**

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Endemisitas penyakit Avian influenza pada itik yang hidup disekitar peternakan ayam petelur komersial (Endemicity of avian influenza in ducks living around commercial layer farms)

(Org: Eng)

JITV 20(4): 285-296

The progenitors of all avian influenza viruses are generally derived from ducks or other waterfowl that have undergone mutation and adaptation to become pathogenic in chickens or other poultry. Investigation of the presence of avian influenza viruses in ducks especially those living around chicken farms is, therefore, important. Serum from 54 ducks and 51 Muscovy ducks living around commercial layer farms in the districts of Cianjur and Sukabumi were collected in March-April 2014. The indication of AI-virus infection in those birds was based on an array of serological tests including competitive and indirect ELISAs for antibody to nucleoprotein, MM2e ELISA for antibody to M2e, HI test, ELISAs and dot blot for antibodies to haemagglutinin, and dot blot assay for antibodies to neuraminidase. Recombinant Haemagglutinins (H1-H13 and H15), recombinant neuraminidases (N1, N2, N7 and N9) and recombinant influenza-A nucleoprotein were used in the indirect ELISAs and dot blot assays. As many as 63% of duck samples and 13% Muscovy-duck samples were serologically positive to nucleoprotein, and 62% of the nucleoprotein-seropositive ducks were also positive to M2e. The high seroprevalence of AI in the ducks living around commercial poultry farms suggested that application of strict biosecurity measures on those farms is still needed. Based on the results of the ELISA and dot blot assays, AI virus subtypes H9N2 and H5N2, in addition to H5N1, were suspected to be circulating in those ducks. Further confirmation by virus isolation, however, is required because H9N2 and H5N2 subtypes have yet been unknown Indonesia and both the subtypes can cause serious disease in poultry.

(Author)

**Key Words:** Duck, Immunoassay, Avian Influenza, H5N1, H5N2, H9N2

**UDC: 303.684**

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Pengembangan teknik identifikasi molekuler spesies *Trypanosoma evansi* dengan *Polymerase Chain Reaction* Multipleks (Molecular identification technique of Trypanosoma evansi by Multiplex Polymerase Chain Reaction)

(Org: Eng)

JITV 20(4): 297-307

*Trypanosoma evansi* is a Hemoflagella parasite that infects cattle and is known as the agents of Surra. Several other trypanosome species infects mammals: *T. equiperdum*, *T. b. rhodesiense*, *T. b. gambiense*, *T. vivax*, *T. congolense*, *T. theileri*. Some of these species is quite difficult to be distinguished morphologically with *T. evansi* through conventional techniques (thin blood smear). Molecular technique by polymerase chain reaction (PCR) is reported to have the ability to identify, characterize and diagnose trypanosomes accurately. However, a single PCR used is relatively expensive because it takes at least two or more pairs of primers to determine *T. evansi*. The purpose of this study is to develop *T. evansi* species identification techniques by multiplex PCR/mPCR (the three pairs of primer in one reaction) that takes the relatively fast and inexpensive. A total of 31 isolates *T.evansi* were obtained from Bblitvet Culture Collection (BCC) and the Department of Parasitology BBLitvet used in this study. Isolates represent isolates from endemic areas and Surra outbrake isolated from 1988-2014. DNA extraction performed on each sample, including Bang 87 isolates which has been purified as a positive control. Primers used are specific for *T. evansi*, the ITS-1, Ro Tat 1.2 VSG and ESAG 6/7. Before running mPCR, each primer is optimized by using a single PCR. The results showed that the three primers can be combined in a single reaction with mPCR technique and amplify each DNA fragment target perfectly, so identified 31 isolates as *T. evansi*. This technique can be applied in the field with a lower cost and faster time.

(Author)

**Key Words**: *Trypanosoma evansi*, Identification, Multiplex PCR

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

**Proceeding:**

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

**Thesis:**

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

**Electronic magazines:**

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

**Institution:**

a. [NRC] National Research Council. 1985. Nutrient requirements of sheep. 6th revised. Washington DC (USA): National Academic Press.

b. [CDC] Centers for Disease Control. 2006. Standard operating procedure for the direct Rapid Immunohistochemistry Test (dRIT) for the detection of rabies virus antigen. [accessed December 20th 2011]. http://www.rabiesblueprint.com/IMG/pdf/DRIT_SOP.pdf.

**Patent:**

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

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