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# The Philippine Journal of Veterinary Medicine

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## CONTENTS

### Original Articles

#### Medicine

- Viability of Rabbit Adipocyte Stem Cells Cultured Under Different Oxygen Concentrations *In Vitro*.....1  
*E Safitri, P Srianto, TV Widiyatno, W Sandhika and RH Prasetyo*

#### Microbiology

- Antigenic Site of Glycoprotein Encoding Gene in Rabies Virus Isolate from Indonesia.....9  
*J Rahmahani, S Suwarno and FA Rantam*
- Characterization of Newcastle Disease Virus Lentogenic Strain Infected Native Chickens from Surabaya, Indonesia.....17  
*FA Rantam, R Ernawati, AP Rahardjo, IL Rahmawati, D Kartika, NS Widjaja and J Rahmahani*

#### Nutrition

- Effect of Concentrate to Forage Ratio on Milk Urea Nitrogen, Milk Production and Reproductive Performance of Dairy Cows.....25  
*S Utama, S Mulyati, W Wurlina and I Mustofa*

#### Pathology

- Toxicity, Stability and Renal Histopathology of Alkaloid of Jarong (*Achyranthes aspera* Linn.) (Caryophyllales: Amaranthaceae) Leaf on Mice.....35  
*DK Meles, W Wurlina, I Mustofa, S Zakaria, A Basori, M Hariadi, E Safitri, DKSC Putri and N Suwasanti*
- Histochemical Expression of Transforming Growth Factor Beta and Tumor Necrosis Factor Alpha in Rabbits Infected with *Sarcoptes scabiei*.....43  
*SM Rizki, LT Suwanti and NDR Lastuti*

#### Pharmacology

- Effect of Alkaloid of *Achyranthes aspera* Linn. (Caryophyllales: Amaranthaceae) on Increasing Caspase 9, Caspase 3 and Apoptosis in Mice with Breast Cancer.....51  
*W Wurlina, DK Meles, I Mustofa, E Safitri, S Zakaria, A Basori, DKSC Putri and N Suwasanti*

#### Theriogenology

- Effect of Aluminum Silicate on the Spermatozoa, Plasma Membrane and Seminiferous Tubules of Mice Exposed to *Fusarium graminearum* (Sordariomycetes: Hypocreales: Nectriaceae).....59  
*Samik, S Mulyati, T Hernawati and E Safitri*

## Research Notes

### Microbiology

Isolation and Identification of Lactic Acid Bacteria from the Digestive Tract of Kampung Chicken (*Gallus gallus domesticus*).....67  
*B Yulianto, WP Lokapirnasari*

*In Vitro* pH Tolerance, Bile Salt Resistance and Antimicrobial Activity of *Lactobacillus plantarum* Isolated from Crossbred Cattle.....73  
*WP Lokapirnasari, AM Sahidu, L Maslachah, K Soepranianondo, AB Yulianto, D Afikasari, TB Pribadi and I Hariyati*

### Nutrition

Amino Acid Sequence of Signal Transducers and Activators Transcription Proteins From Broilers.....79  
*A Ma'ruf, NMR Widjaja, N Hidajati and R Damayanti*

### Parasitology

Antigenic Protein Profile of *Anisakis* spp. Larvae Isolated from Mackerel Tuna Fish (*Euthynnus* sp.).....85  
*ZN Wastomi, NDR Lastuti, R Ernawati, LT Suwanti, S Koesdarto, M Mufasirin and HM Raharjo*

Morphological Detection of the Intestinal Parasite *Blastocystis* sp. in Fresh and Cultured Feces of Pet Sugar Glider (*Petaurus breviceps*) in Surabaya, Indonesia.....91  
*F Natalia, LT Suwanti, E Suprihati, Kusnoto, S Koesdarto and P Srianto*

### Pathology

Comparative Histopathologic Changes in Rabbit (*Oryctolagus cuniculus*) Skin in Relation to Degree of Infestation with *Sarcoptes scabiei*.....97  
*A Azhimah, NDR Lastuti, A Arimbi, D Legowo, P Hastutiek and LR Yustinasari*

### Pharmacology

Effect of Sapogenin from Sambiloto (*Andrographis paniculata*) (Lamiales: Acanthaceae) on Creatinine and BUN Levels and on Gentamicin-Induced Nephrotoxicity in Rats.....103  
*S Zakaria, W Wurlina, DK Meles, I Mustofa, M Hariadi, S Susilowati, E Safitri, A Basori, DKSC Putri and N Suwasanti*

### Public Health

Identification of Shiga Toxin-Producing *Escherichia coli* in Raw Milk Samples from Dairy Cows in Surabaya, Indonesia.....109  
*MH Effendi, N Harijani, SM Yanestria and P Hastutiek*

Tetracycline Resistance Gene in *Streptococcus agalactiae* Isolated from Bovine Subclinical Mastitis in Surabaya, Indonesia.....115  
*MH Effendi, A Oktavianto and P Hastutiek*

### Theriogenology

Bacterial Isolates from the Cervical Mucus of Dairy Cattle at Follicular and Luteal Phases.....121  
*K Sudrajad, SP Madyawati, W Tyasningsih, R Rimayanti, P Srianto and OS Widodo*

|   |            |
|---|------------|
| <b>Human Chorionic Gonadotropin (hCG) from Urine of Pregnant Women for <i>In Vitro</i> Maturation of Madura Cattle Oocytes.....</b> | <b>127</b> |
| <i>HA Hermadi, RTS Adikara, M Hariadi and E Safitri</i>   |            |
| <b>Effect of Bovine Seminal Protein on the Quality of Frozen Spermatozoa from Goats.....</b>  | <b>133</b> |
| <i>S Susilowati, IN Triana, TW Suprayogi, A Arimbi and W Wurlina</i>  |            |
| <b>Editorial Policies.....</b>  | <b>139</b> |
| <b>Guidelines for Authors.....</b>  | <b>141</b> |

**RESEARCH NOTE****BACTERIAL ISOLATES FROM THE CERVICAL MUCUS OF DAIRY CATTLE AT FOLLICULAR AND LUTEAL PHASES**

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**ABSTRACT**

The research aimed to identify the bacteria present in the reproductive tract of dairy cattle at follicular and luteal phases of the estrus cycle. Seven samples of cervical mucus were each obtained from the reproductive tract, at follicular and luteal phase, of fourteen healthy cattle, 2-3 years of age, with body condition scores of 2.0-8.3. Sporet, motility, triple sugar iron agar, mannitol and glucose tests were done to obtain gram characteristics and bacterial morphology, which enabled identification at the genus level. Non-specific bacteria isolated at follicular phase were *Staphylococcus* (50%), *Corynebacterium* (25%) and *Escherichia* (25%). At luteal phase, *Staphylococcus* (45.5%), *Escherichia* (45.5%) and *Corynebacterium* (9%) were isolated. The results suggest that a number of bacteria can be found in the reproductive tract of dairy cattle, both at follicular phase and luteal phase.

**Key words:** bacteria, dairy cattle, follicular phase, luteal phase, reproductive tract

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**INTRODUCTION**

Dairy cattle farms in Indonesia run by natives are small-scale and typically rely on conventional breeding system. In many cases, reproductive disorders arise from lack of attention to the overall health of cattle, which directly influence their reproductive status. Reproductive performance is essential to well performing and profitable dairy farms (Nebel and Jobst, 1998), and this is directly or indirectly influenced by many factors. Cow infertility, for instance, is affected by many specific and non-specific pathogens of the genital tract. The cervix and its secretions play a vital role in the reproductive performance of mammals (Blandau and Moghissi, 1973).

Cervical mucus discharge (CMD) is a mechanical barrier against pathogens of the uterus. Normally, an estrous cow discharges a viscous liquid from the vulva. The healthy liquid is clear, originates from the cervix and has no bad odor. Clear CMD on the artificial insemination (AI) gun, following insemination, is positively associated with an increased first service conception rate (FS-CR) (Loeffler *et al.*, 1999).

Basically, bacterial infection in cattle disturbs or attacks reproductive organs, especially the uterus. Uterus infection can be caused by non-specific or specific bacteria (Hafez and Hafez, 2004). At follicular phase, estrogen level tends to increase in the blood, inhibiting bacterial growth; whereas, at luteal phase, higher progesterone level is

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pronounced. Hence, hormonal differences in both phases need to be observed to differentiate bacterial types that predominate in dairy cattle reproductive system.

Koperasi Serba Usaha (KSU) Tunas Setia Baru Tutur sub-district Pasuruan Regency is a cooperative that runs dairy cattle business in East Java, Indonesia, mainly producing fresh milk. Located at the slope of western Tengger Mountain, with a latitude of 400-2000 m, working areas of KSU Tunas Setia Baru cover 10 villages in the Tutur sub-district, Pasuruan. Currently, KSU uses information technology to develop the business in cooperation with government and local organizations.

Since reproductive health plays a huge role in cattle productivity, this research aims to identify non-specific bacteria in the reproductive tract, at follicular and luteal phase, of female dairy cattle from KSU Tunas Setia Baru Tutur sub-district Pasuruan Regency.

## MATERIALS AND METHODS

### Sample collection

This study used 14 healthy Friesian Holstein dairy cattle, 2-3 years of age, collected from KSU Tunas Setia Baru Tutur, Pasuruan. Animals were equally divided into two groups, taking seven samples of cervical mucus at follicular and luteal phase. A plastic sheath, used as a veil of insemination gun during artificial insemination, was used to collect cervical mucus samples. This was cut 2-3 cm from the tip, placed in a tube with PBS medium and kept in an icebox at 4°C. Experiments were performed at the Bacteria and Mycology Laboratory of Microbiology Department, Faculty of Veterinary Medicine, Universitas Airlangga, from April to July 2017.

### Bacterial isolation

Samples kept in PBS media were planted on tripticase soya agar (TSA) and blood agar (BA). To isolate the bacterial genus *Staphylococcus* and *Escherichia*, selective media mannitol salt agar (MSA) and eosin methylene blue agar (EMBA) were used. Each medium was incubated for 24 h. The isolates

were then subjected to gram staining using standard procedures (John *et al.*, 1994).

### Catalase test

This test was done to determine the activity of the catalase enzyme, so that different colonies that formed can be identified. This was conducted by dripping 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on glass slide. Culture was then smeared on glass slide. Bubble formation indicates positive result. This was conducted on gram-positive bacteria with coccus morphology. Presence of bubbles or froth signifies that a sample is a catalase-positive *Staphylococcus*. Otherwise, it is a catalase-negative *Streptococcus* (Chester, 1979).

### Spore test and motility test

Spore test was carried out to determine whether tested bacteria can form spores or not, given that *Corynebacterium* are unable to form spores. This was done through a heating process using malachite green and was only applied to gram-positive bacteria with bacillus morphology (Bergey and Holt, 1994). Motility test was done via physical examination to determine bacterial motility.

## RESULTS AND DISCUSSION

Both gram-positive and gram-negative bacteria were identified from the cervical mucus of female dairy cattle reproductive tract (Fig.). During the study, none of the cows exhibited obvious signs of disease. The most frequently isolated bacteria were gram-positive. Results revealed that, at follicular phase, there were gram-positive bacteria (77%) indicated by purple colonies, while gram-negative bacteria (23%) were those with red colonies. At luteal phase, gram-positive bacteria were as much as 58%, while gram-negative bacteria accounted for 42%. Details are presented on Tables 1 and 2.

Catalase test was applied only to gram positive cocci, *i.e.*, on samples 3, 4, 5 and 7 at follicular phase and samples 1, 3, 5, 6 and 7 at luteal phase. Results showed four positive colonies at follicular phase and five at luteal phase (Table 3). Spore test done on bacillus



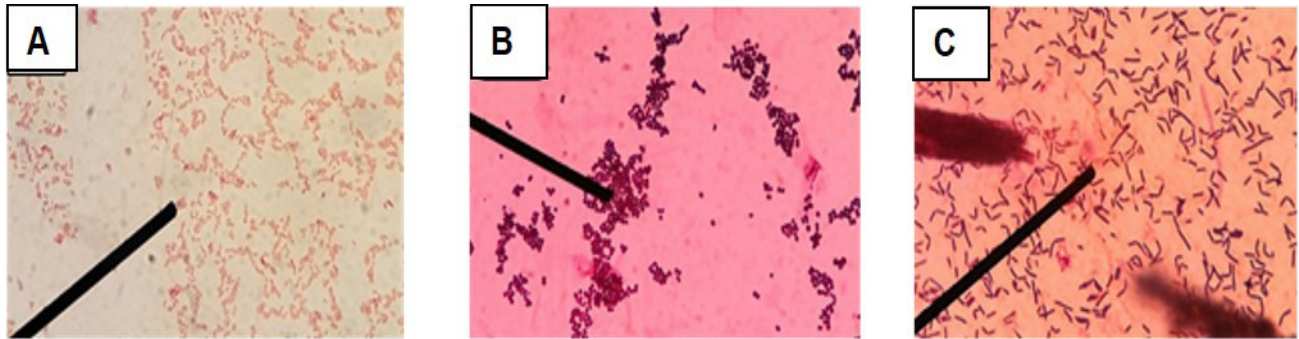


Fig. Morphology of gram-stained bacteria isolated from the reproductive tract of dairy cattle under an optic light microscope at 100× magnification. A: gram-negative coccobacillus; B: gram-positive coccus; C: gram-positive bacillus.

Table 1. Gram staining results of cervical mucus samples isolated from the reproductive tract of dairy cattle at follicular phase.

| Sample | Morphology   | Gram staining<br>Colony color | Result |
|--------|--------------|-------------------------------|--------|
| 1      | bacillus     | purple                        | +      |
|        | cocobacillus | red                           | –      |
| 2      | bacillus     | purple                        | +      |
|        | coccus       | purple                        | +      |
| 3      | bacillus     | purple                        | +      |
|        | coccus       | purple                        | +      |
| 4      | bacillus     | purple                        | +      |
|        | cocobacillus | red                           | –      |
| 5      | coccus       | purple                        | +      |
| 6      | coccus       | purple                        | +      |
|        | bacillus     | purple                        | +      |
| 7      | coccus       | purple                        | +      |
|        | cocobacillus | red                           | –      |

Table 2. Gram staining results of cervical mucus samples isolated from the reproductive tract of dairy cattle at luteal phase.

| Sample | Morphology   | Gram staining<br>Colony color | Result |
|--------|--------------|-------------------------------|--------|
| 1      | coccus       | purple                        | +      |
|        | cocobacillus | red                           | –      |
| 2      | bacillus     | purple                        | +      |
|        | coccus       | purple                        | +      |
| 3      | cocobacillus | red                           | –      |
|        | cocobacillus | red                           | –      |
| 4      | coccus       | purple                        | +      |
|        | bacillus     | purple                        | +      |
| 5      | cocobacillus | red                           | –      |
|        | cocobacillus | red                           | –      |
| 6      | bacillus     | purple                        | +      |
|        | coccus       | purple                        | +      |
| 7      | coccus       | purple                        | +      |

gram-positive bacteria produced two negative colonies at follicular phase and one at luteal phase (Table 4).

Bacterial identification in cervical mucus samples in dairy cattle detected the following number of colonies at follicular phase: four *Staphylococcus*, two *Escherichia* and two *Corynebacterium*. In luteal phase, there were five *Staphylococcus*, five *Escherichia* and one *Corynebacterium* (Table 5).

This study shows that there is no distinct difference on the bacterial population, at the genus level, between phases. In fact, both were defined by similar non-specific bacteria from the genus *Staphylococcus*, *Corynebacterium* and *Escherichia*. Genus *Staphylococcus*, normally found on the surface of skin, was mostly found either at follicular or luteal phase. This group is assumed to enter the reproductive tract due to direct or indirect contact with skin. Another possibility of entry through the uterus may be traced from misconduct of artificial insemination procedure or failure to follow procedures on correction of dystocia. Success

in isolating *Staphylococcus* from normal cattle uterus is possible when the animal is weak, or when there is wound in the uterus mucosa. *Staphylococcus aureus* causes one of the most common types of chronic mastitis. Though some cows may flare up with clinical mastitis (especially after calving), the infection is usually subclinical, causing elevated somatic cell counts but no detectable changes in milk or the udder. (Petersson *et al.*, 2010).

*E. coli* normally live in the guts of humans and animals (Magiorakos *et al.*, 2012). However, they can also proliferate in the reproductive tract. In this study, *Escherichia* found in the reproductive tract are assumed to be due to the presence of feces (anatomically, anus is close to external genitals). Vaginal bacterial flora in cows affected by metritis are dominated by strains of *E. coli* (Wang *et al.*, 2013). Williams *et al.* consider high cell counts of *E. coli* as basis for the onset of uterine infection.

*Corynebacterium* population found in the study existed on the ground, in water,

Table 3. Results of catalase test on coccus gram-positive bacteria isolated from the reproductive tract of dairy cattle at follicular and luteal phase.

| Sample | Follicular phase | Luteal phase |
|--------|------------------|--------------|
| 1      | NA               | +            |
| 2      | NA               | NA           |
| 3      | +                | +            |
| 4      | +                | NA           |
| 5      | +                | +            |
| 6      | NA               | +            |
| 7      | +                | +            |

Table 4. Results of spore test on bacillus gram-positive bacteria isolated in the reproductive tract of dairy cattle at follicular and luteal phase.

| Sample | Follicular phase | Luteal phase |
|--------|------------------|--------------|
| 1      | +                | NA           |
| 2      | -                | +            |
| 3      | -                | NA           |
| 4      | +                | +            |
| 5      | NA               | -            |
| 6      | +                | +            |

Table 5. Results of bacterial identification of cervical mucus samples isolated from the reproductive tract of dairy cattle at follicular and luteal phase.

| Follicular phase |            |                        | Luteal phase |            |                        |
|------------------|------------|------------------------|--------------|------------|------------------------|
| Sample           | Gram stain | Genus                  | Sample       | Gram stain | Genus                  |
| 1                | -          | -                      | 1            | +          | <i>Staphylococcus</i>  |
|                  |            |                        |              | -          | <i>Escherichia</i>     |
| 2                | +          | <i>Corynebacterium</i> | 2            | -          | -                      |
| 3                | +          | <i>Staphylococcus</i>  | 3            | +          | <i>Staphylococcus</i>  |
|                  | +          | <i>Corynebacterium</i> |              | -          | <i>Escherichia</i>     |
| 4                | +          | <i>Staphylococcus</i>  | 4            | -          | <i>Escherichia</i>     |
|                  | -          | <i>Escherichia</i>     |              | +          | <i>Staphylococcus</i>  |
| 5                | +          | <i>Staphylococcus</i>  | 5            | +          | <i>Corynebacterium</i> |
|                  |            |                        |              | -          | <i>Escherichia</i>     |
| 6                | -          | <i>Escherichia</i>     | 6            | +          | <i>Staphylococcus</i>  |
| 7                | +          | <i>Staphylococcus</i>  | 7            | -          | <i>Escherichia</i>     |
|                  |            |                        |              | +          | <i>Staphylococcus</i>  |

and in pasture consumed by the cattle. This genus is a non-specific bacteria that can cause a reproductive disorder called endometritis (Bage *et al.*, 2002). Cattle might have caught the organism during artificial insemination or through unhygienic birth handling, which might have allowed non-specific bacteria to enter and proliferate (Baya *et al.*, 1992).

Uterine infections in dairy cows are associated with predisposing factors, including calving difficulty, retained placenta, compromised immune status and parity, along with overgrowth of pathogenic microorganisms in the reproductive tract (Coleman *et al.*, 1985). There are several factors that directly or indirectly influence the reproductive performance of cows. For instance, many specific and non-specific pathogens of the genital tract affect cow infertility. To protect against these pathogens, mammals rely on the roles of the cervix and its secretions (Blandau and Moghissi, 1973).

Metritis-associated bacteria can be pathogens, potential pathogens or opportunistic pathogens (Sheldon *et al.*, 2002). Recognized uterine pathogens associated with severe endometrial inflammation and clinical endometritis include *Escherichia coli*, *Arcanobacterium pyogenes*, *Fusobacterium necrophorum*, *Prevotella melaninogenica* and

*Proteus* species. Bacteria found within the microbiota are thus likely to be contaminants from the environment (*Bacillus* spp.), cow's skin (*Staphylococcus* spp.) or fecal material (*E. coli*, lactic acid bacteria), rather than representing a stable flora autochthonous to the reproductive tract (Williams *et al.*, 2007).

The lack of a competitive commensal vaginal microbiota may contribute to the susceptibility of dairy cows to bacterial overgrowth and metritis after parturition. Bacilli are present in the environment, and they frequently contaminate the bovine uterine lumen (Williams *et al.*, 2008). Consequently, one way by which farmers and artificial inseminators address this susceptibility to pathogens is by monitoring the cervical mucus discharge of estrous cows and heifers with abnormal appearance (Mahmoudzadeh *et al.*, 2001).

This study shows that there is no difference in the bacterial population in the reproductive tract of dairy cattle detected at both phases, follicular and luteal. There were three non-specific bacterial genus identified - *Staphylococcus*, *Escherichia* and *Corynebacterium*. At follicular phase, genus *Staphylococcus* accounted to as much as 50%, *Corynebacterium* to 25% and *Escherichia* to 25%. At luteal phase, *Escherichia* shared as

much as 45.5%, *Staphylococcus* as at 36.4% and *Corynebacterium* at only 0.1%.

This study recommends improving overall sanitary management in livestock and training inseminators on strict hygienic practices to prevent the development of reproductive diseases in dairy cattle.

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