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RESEARCH NOTE

COMPARATIVE HISTOPATHOLOGIC CHANGES IN RABBIT (*Oryctolagus cuniculus*) (MAMMALIA: LAGOMORPHA: LEPORIDAE) SKIN IN RELATION TO DEGREE OF INFESTATION WITH *Sarcoptes scabiei* (ARACHNIDA: ACARI: SARCOPTIDAE)

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ABSTRACT

The aim of this research was to observe the histopathological changes in rabbit ear skin tissue caused by varying degrees of *Sarcoptes scabiei* infestation. This study used twelve 7-12 months old female local rabbits obtained from rabbit farms with poor sanitation. Clinical symptoms of ear infection include presence of papules, vesicles, erythema, crusta and alopecia in the ear, nose, eyes and feet. Twelve local rabbits infected by *S. scabiei* were divided into three groups with four rabbits each: P1 with mild scabies, P2 with moderate scabies and P3 with severe scabies. Histopathologic changes, which manifested in lesions, varied from parasitic infestation, parakeratosis, acanthosis, congestion, inflammation and cell degeneration. These were given scores from 0 to 4 (0, not seen; 4, highly visible). The mean score was highest in those with severe scabies. Histopathological changes in rabbit ear skin tissue using Mann-Whitney U test was significant ($P<0.05$): mild scabies (4.625 ± 0.75), moderate scabies (8.8125 ± 1.95) and severe scabies (17.5625 ± 1.59). Severe scabies had the highest degree of damage, defined by parakeratosis, acanthosis, substantial cell degeneration and congestion and serious inflammation. This study suggests significant differences in histopathologic changes in skin tissue of rabbits with mild, moderate and severe scabies.

Key words: histopathologic changes, rabbit, *Sarcoptes scabiei*, scabies

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INTRODUCTION

To this day, scabies has been considered an emerging or re-emerging parasitic disease that threatens human and animal health worldwide because of its prevalence, and ways to control its spread are still limited, resulting to major economic losses (Tarigan, 2003; Walton and Currie, 2007; Alasaad *et al*., 2013). Several areas of goat and rabbit farming in Indonesia have been reported for scabies infection, most likely caused by poor hygiene and sanitation. In addition, crammed cages and humidity contribute to the transmission of scabies to healthy animals (Wardhana *et al*., 2006; Lastuti *et al*., 2017). Scabies can be diagnosed by looking for clinical signs and performing laboratory tests, such as microscopic examination of skin scraping or through burrow ink test (Budiantono, 2004; Walton and Currie, 2007). Research in scabies diagnosis has been developed, and findings suggest that *S. scabiei* var. *caprae* in goats

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contains specific antigen protein around 205, 8, 57, 3 and 43 kDa, which can then be used to develop a candidate diagnostic kit (Lastuti *et al.*, 2018). *Sarcoptes scabiei* is a parasite that requires a host to breed, and, once settled, creates tunnels in the stratum corneum, sucking the lymphatic fluid by tearing the epidermal layer and feeding on young epidermal cells. This causes intense itching and can lead to injury when the skin is scratched persistently, releasing an exudate which causes skin scabs (Soulsby, 1986; Espinosa *et al.*, 2017).

If scabies infection reaches the connective tissue, along with skin fibrosis, epidermal hyperplasia and mononuclear cell become dominant in the perivascular, reaching chronic stage, which can be detrimental to the animal (Budiantono, 2004; Arlian *et al.*, 2004; Espinosa *et al.*, 2017). Determining the extent of skin damage caused by mild to severe scabies through histopathologic changes can be important, since chronic inflammation can lead to economic losses in rabbit livestock. Also, determining this can serve as reference for scabies control.

The aim of this research is to explore the extent of damage to rabbit skin tissue infected with mild to severe scabies based on histopathologic changes. Assessing these changes by scoring method will be done for the first time in Indonesian samples. This study assumes significant differences in histopathologic changes in rabbit skin tissue with varying degrees of scabies infection. Reaching a certain threshold, *e.g.*, displaying severe clinical symptoms can be an important factor when deciding to eliminate animals in a population. The results of this study can then be used as a reference for scabies prevention in rabbits.

MATERIALS AND METHODS

Animals

This research was approved by the Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga, No: 630-KE in accordance with the rules of experimental animal use. This research was done in the Veterinary Pathology Laboratory and Veterinary Parasitology Laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga. Animals used were 12 female, 7 to 12 months old local rabbits infected with scabies, divided into three groups with four rabbits each (P1 with mild scabies, P2 with moderate scabies and P3 with severe scabies). Sampling criteria were based on a number of clinical signs: mild scabies has papules, vesicles, erythema and few crusta on the ear; moderate scabies shows mild hyperkeratosis or crusts on the ear, mild alopecia and thin scabs; meanwhile, severe scabies is characterized by the presence of crusts, pus and excessive lichenification, which causes the skin to look wrinkly and cracked, along with alopecia in almost all of the infected skin area (Espinosa *et al.*, 2017).

Identification of *S. scabiei* var. *cuniculi*

To verify scabies infection, the ears were scraped then added with 10% KOH (Merck, Germany), and samples were examined under a microscope (Nikon E-100, Japan) at 100× magnification. *Sarcoptes scabiei* was identified using identification keys by Soulsby (1986). After being tested positive for scabies, the rabbits were euthanized by injecting 100 mg/kg ketamine (Mylan, Singapore) intramuscularly. Infected ears were incised with a diameter of 1 cm × 1 cm, and skin tissues were then immersed in 10% PBS solution (Merck, Germany) for histopathology preparations.

Preparation of histopathology specimens

Samples were fixed using 10% PBS solution, soaked for 24 h and washed with distilled water. Samples were dehydrated and cleared with alcohols 70%, 80%, 96%, absolute I-III, and xylol I and II (Merck, Germany) for 30 min. Tissues were submerged into paraffin I and II fluids, put into the oven (Memmert, Germany) at 80°C for 30 min, dipped back into paraffin fluids, then into the oven at same conditions. Paraffin blocks were made. Tissues that have expanded adequately after being dipped into warm water at 60°C were sliced with a thickness of 4-6 μm. These samples were then placed on a glass object (Sail Brand, China) previously smeared with

albumin, then dried over a hot plate at 60°C, and stained with hematoxyline eosine (HE) (Merck, Germany). Histological examination was done using a microscope (Nikon E-100, Japan) at magnifications 40×, 100×, 400×, followed by assignment of scores from 0 to 4 (0, not seen; 4, highly visible) on each lesion in terms of parasitic infestation, parakeratosis, acanthosis, cell degeneration, congestion and inflammation (Klopfleisch, 2013). Scoring results were analyzed using statistical tests Kruskal Wallis and Mann-Whitney U Test.

RESULTS AND DISCUSSION

Scraping of infected rabbit skin showing scabies symptoms identified the parasitic mite *S. scabiei* var. *cuniculi*. Histopathological changes in the skin tissue infected with scabies of varying severity, from mild, moderate and severe, were evident in the epidermis, defined by parakeratosis and acanthosis, and the dermis, characterized by parakeratosis, inflammatory cell infiltration, degeneration and congestion (Fig).

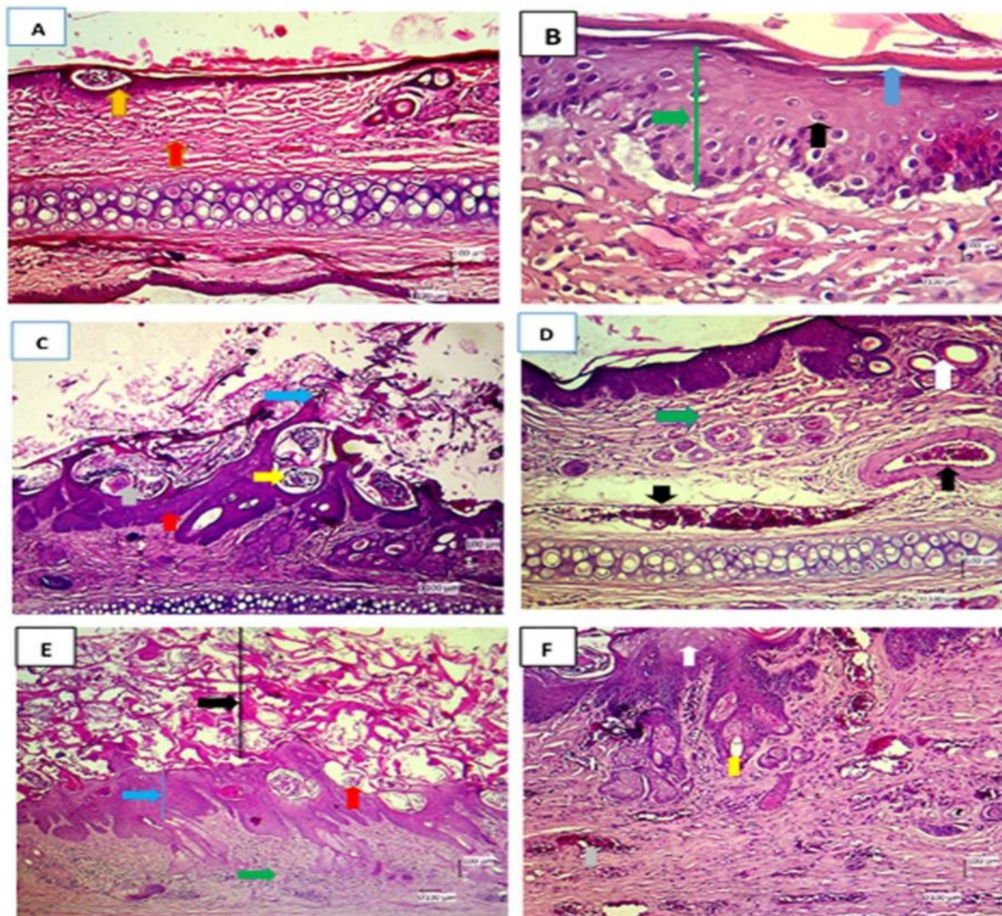

Fig. A and B: Histopathologic changes seen in rabbit with mild scabies: (yellow arrow) infestation of *S. scabiei,* (red arrow) inflammatory cell infiltration, (blue arrow) thin hyperkeratosis, (green arrow) acanthosis, (black arrow) cell degeneration. C and D: Histopathologic changes in rabbit with moderate scabies: (blue arrow) parakeratosis, (yellow arrow) mature mites, (gray arrow) mite larvae, (red arrow) acanthosis, (green arrow) infiltration of inflammatory cells, (white arrow) alopecia, (black arrow) congestion. E and F: Histopathologic changes in rabbit with severe scabies: (black arrows) severe parakeratosis, (blue arrows) acanthosis, (red arrows) mature infestation of mites, (green arrows) inflammatory cell infiltration, (white arrow) cell degeneration, (yellow arrow) alopecia, (gray arrow) congestion. (Bar = 100 µm) Nikon® E-100 microscope.

Histopathological changes in the rabbits were evaluated based on the descriptions of parakeratosis, acanthosis, congestion, inflammation and degeneration of cells. These were given scores of 0-4 (Klopfleisch, 2013). Results of the mean scores are presented in Table 1. Kruskal Wallis test, followed by Mann-Whitney U test, indicates a significant difference (P<0.05) between groups (Table 2).

Histopathologic changes induced by parasitism caused an immune response (protection against foreign agents) and disturbances in keratinization, in the form of parakeratosis and acanthosis. Keratinization, in particular, has resulted to thickened and layered skin and hair loss, leading to secondary infection (Espinosa *et al*., 2017). Based on its life cycle, *S. scabiei* begins its settlement by penetrating and sucking on the lymph, feeding on young epidermal cells, which then causes irritation and intense itching. Scratching leads to formation of a solid exudate and a crust on the skin's surface. Clinical symptoms of severe itching seem to be associated with type I, III and IV hypersensitivity reactions in humans (Arlian *et al*., 2004), and it appears that *S. scabiei* creates a substance that activates type 1 T-cells to produce IL-10, which plays anti-inflammatory and immune suppression roles (Arlian *et al*., 2004; Lastuti *et al*., 2018).

Table 1. Mean scores of histopathologic changes in *S. scabiei* var. *canaliculi* based on degree of infestation in rabbit.

Parameter	Mean score		
	Mild scabies	Moderate scabies	Severe scabies
No. of parasites	0.3	1.4	3.4
Parakeratosis	0.3	0.9	2.6
Acanthosis	0.5	1.6	3.5
Cell degeneration	1.7	2.2	3.2
Congestion	0.4	0.6	1.0
Inflammation	1.4	2.1	3.8

Table 2. Statistical test scores of histopathological changes in *S. scabiei* var. *canaliculi* based on degree of infestation in rabbit.

	Mild scabies	Moderate scabies	Severe scabies
Total score (mean±SD)	4.625±0.7500[c]	8.812±1.9512[b]	17.562±1.5898[a]

Different superscripts on the same column show significant difference (P<0.05).

Mitosis in the epidermal layer starts from the basal stratum which increases the production of keratin cells rapidly, resulting to acanthosis, the thickening of the stratum spinosum due to increased cell division. In severe scabies, parakeratosis and acanthosis occur very significantly compared with moderate and mild scabies due to increased infestation by *S. scabiei*. (Nanney *et al*., 1986; Espinosa *et al*., 2017). Parakeratosis, characterized by the presence of of pyknotic nuclei that aggregate in the stratum corneum, occurs since the epidermal cells fail to differentiate completely. Further change in the structure of the epidermis caused by *S. scabiei* infestation is injury to the cell membrane of epithelial cells, especially in the granular layer and stratum spinosum. This decreases the cell's permeability because of the antigenic proteins released by the mites

during penetration, as the parasites suck on blood and fluid lymph and feed on epidermal cells. Such conditions allow K^+ ions to be easily transported outside the cells and vice versa; Ca^+, Na^+ and water easily pass through the cells, causing the cytoplasm to swell (Hennings *et al*., 1983).

Hydropic degeneration that attacks epithermal epithelial cells in severe scabies is more significant compared with moderate and mild scabies, since higher antigen production means reduced cell permeability and formation of hydropic degeneration in epidermal epithelial cells. Lesion on the epidermal layer will then activate the inflammatory mediator, which stimulates the vasoactive amine, increasing vascular permeability, vasodilation and activation of histamine and serotonin. Blood flow out of the veins is reduced, and blood accumulates in the vein, a condition called congestion (Charles *et al*., 1967).

Accumulation of erythrocytes also influences the the movement of leukocytes, becoming attached to the edge of the endothelium in an attempt to exit the site of lesion. There were significant differences in congestive veins in severe, moderate and mild scabies. High inflammatory reaction in severe scabies occurs because of higher degree of infestation, leading to increased blood flow, vasodilation and congestion.

Prolonged and worsened *S. scabiei* infestation will activate type IV hypersensitivity reaction due to tissue damage from the accumulation of macrophages, monocytes and lymphocytes in exposed areas (Walton *et al*., 2010; Singh *et al*., 2014; Lastuti *et al*., 2018). Skin tissue of rabbits with severe *S. scabiei* infestation shows evidence of chronicity: many mononuclear cells dominated the surface of the dermis, and the perivascular was inflamed; meanwhile, polymorphonuclear cells predominated in the dermis layer of rabbits with moderate and mild scabies. Morever, other histopathologic changes include alopecia (hair loss in animals), a condition which disrupts the body's immune system due to infection caused by parasites, viruses, bacteria or stress (Bandi and Saikumar, 2013; Kuty-Pacheck, 2015; Espinosa *et al*., 2017).

This study reveals significant differences in histopathological changes in rabbit skin tissue with mild, moderate and severe scabies based on the presence of parakeratosis, acanthosis, substantial cell degeneration and congestion, and high level of inflammation. Further studies through immunochemistry is suggested to investigate cytokines in damaged skin caused by scabies.

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REFERENCES

Alasaad S, Rossi L, Heukelbach J, Perez J, Hamarsheh MO, Otiende M and Zhu XQ. 2013. The neglected navigating web of the incomprehensibly emerging and re-emerging sarcoptic mite. *Infection, Genetic and Evolution* 17: 253-259.

Arlian LG, Morgan MS and Cassandra P. 2006. Evidence that scabies mites (Acari: Sarcoptidae) influence production of interleukine-10 and the function of T-regulatory cells (Tr1) in humans. *Journal of Medical Entomology* 43: 283-287.

Arlian LG, Morgan MS, Estes SA, Walton SF, Kemp DJ and Currie BJ. 2004. Circulating IgE in patients with ordinary and crusted scabies. *Journal of Medical Entomology* 41(1): 74-77.

Bandi KM and Saikumar C. 2013. Sarcoptic mange: zoonotic ectoparasitic skin disesase. *Journal of Clinical and Diagnostic Research* 7(1): 156-157.

Budiantono. 2004. Economic losses due to scabies and difficulties in its eradication. *Proceedings of Parasitology Seminar and Veterinary Toxicology* 47-48.

Charles G, Cochrane MD and David HMD. 1967. Studies on circulating immune complexes III. Factors governing the ability of circulating complexes to localize in blood vessels. *Journal of Experimental Medicine* 127(1): 137-154.

Espinosa J, Ráez-Bravo A, López-Olvera JR, Pérez JM, Lavín S, Tvarijonaviciute A,

Cano-Manuel FJ, Fandos P, Soriguer RC, Granados JE, Romero D and Velarde R. 2017. Histopathology, microbiology and the inflammatory process associated with *Sarcoptes scabiei* infection in the Iberian ibex, *Capra pyrenaica*. *Parasite and Vectors* 10: 596.

Hennings H, Holbrook KA and Yuspa SH. 1983. Potassium mediation of calcium induced terminal differentiation of epidermal cells in culture. *The Journal of Investigative Dermatology* 81(1): S50-S55.

Klopfleisch R. 2013. Multiparametric and semi quantitative scoring systems for the evaluation of mouse model histopathology – a systematic review. *BMC Veterinary Research* 9: 123.

Kuty-Pacheck M. 2015. Psychological and psychopathological factors in alopecia areata. *Psychiatria Polska* 49(5): 955-964.

Lastuti NDR, Rantam FA, Hastutiek P and Chrismanto D. 2017. Toll-like receptors (TLRs) play a role in adaptive immunity in rabbits immunized by *Sarcoptes scabiei* proteins. *KnE Life Sciences* 1-9.

Lastuti NDR, Yuniarti WM, Hastutiek P, Suwanti LT and Chrismanto D. 2018. Humoral and cellular immune response induced by antigenic protein of *Sarcoptes scabiei* var. *caprae*. *Veterinary World* 11(6): 819-823.

Nanney LB, Stoscheck CM, Magid M and King LE Jr. 1986. Altered [125I] epidermal growth factor binding and receptor distribution in psoriasis. *The Journal of Investigative Dermatology* 86(3): 260-265.

Singh SK, Dimri U, Sharma B, Saxena M and Kumari P. 2014. Assessment of the cytokine profile in peripheral blood mononuclear cells of naturally *Sarcoptes scabiei* var. *canis* infected dogs. *Veterinary Parasitology* 206 (3-4): 253-257.

Soulsby EJL. 1986. *Helminths, Arthropods and Protozoa of Domesticated Animals* (7th ed.). London: Bailliere Tindall, Ltd.

Tarigan S. 2003. Histopathological changes in naïve and sensitised goats caused by *Sarcoptes scabiei* infestation. *Indonesian Journal of Animal and Veterinary Sciences* 8(2): 114-121.

Walton SF and Currie BJ. 2007. Problems in diagnosing scabies, a global disease, in human and animal populations. *Clinical Microbiology Reviews* 20(2): 268-279.

Walton SF, Pizzutto S, Slender A, Viberg L, Holt D, Hales BJ, Kemp DJ, Currie BJ, Rolland JM and O'Hehir R. 2010. Increased allergic immune response to *Sarcoptes scabiei* antigens in crusted versus ordinary scabies. *Clinical and Vaccine Immunology* 17(9): 1428-1438.

Wardhana J, Manurung T and Iskandar. 2006. Scabies: the challenges of current and future zoonotic diseases. *Wartazoa* 16(1): 40-52.

RESEARCH NOTE

IDENTIFICATION OF SHIGA TOXIN-PRODUCING *Escherichia coli* IN RAW MILK SAMPLES FROM DAIRY COWS IN SURABAYA, INDONESIA

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ABSTRACT

The purpose of this research was to identify the presence of shiga toxin-producing *Escherichia coli* (STEC) in raw milk samples in Surabaya dairy cows using Multiplex Polymerase Chain Reaction (MPCR) assay. Approximately 10 ml milk samples from 75 apparently healthy Holstein Friesian cows from Surabaya, Indonesia were analyzed. Milk samples were inoculated with brilliant green bile broth (BGBB), subcultured in eosin methylene blue agar (EMBA) and were confirmed biochemically using Indol test. Multiplex PCR using primer *flicH7* and primer *stx2* (gene coding shiga toxin) were then performed. Results showed that 26 out of 75 samples were *E. coli* in bacterial isolation and MPCR. Moreover, two samples (7.7%) were positive for *stx2* gene. The MPCR assay described in the present study can be employed to identify and screen for *E. coli* harboring *stx2* gene in raw milk from dairy cows in Indonesia.

Key words: *Escherichia coli,* multiplex PCR, shiga toxin, *stx2* gene

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INTRODUCTION

Raw milk can be a major potential source of harmful bacteria to humans. Outbreaks of foodborne diseases have been reported due to consumption of contaminated raw milk and raw milk products in Indonesia (Suwito, 2010). *E. coli* is one of the most important pathogenic bacteria, which are normal inhabitants of the colon in humans and animals (Hassan *et al.*, 2014). It can be transmitted to raw milk and dairy products due to fecal contamination during milking process and poor hygienic practices (Hogan and Smith, 2003). Milk, while still in the mammary gland of healthy animals, is said to be sterile but susceptible to contamination once released from the udder. Potential sources of contamination in milk are milk cans, cages, farm environment, cow fur, dung, feeds, milking equipment and workers. Contamination may also occur during storage, transportation, distribution, marketing and selling.

Many studies show that *E. coli* O157:H7 is a bacterium that often contaminates milk. It is a pathogenic strain of enterohemorrhagic *Escherichia coli* (EHEC) in humans. The pathogenic nature of *E. coli* O157:H7 is derived from shiga toxins, which can cause hemorrhagic colitis characterized by bloody diarrhea in humans. Shiga toxin from *E. coli* O157:H7 (STEC) is encoded by certain genes possessed by bacteria in the form of the *stx2* gene. The *stx2* gene is one of the major virulence factors of *E. coli* O157: H7 (Andriani,

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2005) that may cause fluid accumulation in the intestines and result to diarrhea.

At present, very little information is available on *E. coli* O157:H7 *stx2* gene from raw milk samples in Indonesia. Therefore, this study was conducted to detect STEC in raw milk samples from Surabaya, Indonesia using Multiplex Polymerase Chain Reaction (MPCR) assay.

MATERIALS AND METHODS

Sampling

Purposive sampling of dairy cows in Subaraya, Indonesia was performed based on specific criteria (Effendi *et al.,* 2017): (a) poor sanitation; (b) inadequate implementation of overall cleanliness and practice of proper hygiene by the farm hands; and (c) unkempt and poorly maintained blade renderers and cages.

A total of 75 dairy cows from four farms in Subaraya were identified. Approximately 10 ml milk samples from each cow were taken directly from the udders and placed into sterile reaction tubes. Milk samples were covered with sterile cotton and inserted into a thermos (ice box) for transport. Milk sampling was performed in the morning from 4 to 6 AM.

Bacterial isolation

Each milk sample was inoculated in BGBB media (E. Merck, Darmstadt, Germany) and incubated at 37ºC for 18-24 h. Positive samples on BGBB, characterized by color change and the presence of gas in the Durham tube, were subcultured in eosin methylene blue agar (EMBA) media (E. Merck, Darmstadt, Germany) and incubated at 37ºC for 18-24 h. Confirmed *E. coli* isolates were subsequently cultured in 5 ml lactose broth and incubated at 37ºC for 48 h for storage and multiplex PCR (Effendi *et al.*, 2017).

DNA extraction

Confirmed *E. coli* isolates in 5 ml lactose broth were centrifuged at 5000 rpm for 10 min. The filtrate was removed and sediments were washed using 5 ml PBS and centrifuged at 5000 rpm for 10 min. Washing was repeated thrice. The sediments were then transferred into microtubes. DNA extraction using DNAzol® Direct reagent was performed. A total of 100 µl TE buffer (10 mm of Tris HCl, 1 mm of ethylene diamine tetraacetic acid (EDTA), pH 8.0) was added to each microtube, followed by the addition of 5 µl lysostaphin enzyme. The mixture was incubated for 1 h at 37ºC and treated with 10 µl proteinase K for 2 h at 56°C. The mixture was boiled for 10 min and cooled in ice for 2 min. Centrifugation was performed at 13000 rpm for 3 min. Supernatant was used for PCR (Effendi, 2010).

Multiplex polymerase chain reaction (MPCR)

The MPCR mixture was prepared using 1.7 µl of primers; 1 µl DNTPs, 5 × 10X thermophilic 2 buffers (Promega), 3 µl MgCl2, 0.2 µl Taq polymerase, 29.8 µl of sterile distilled water and 4.2 uL DNA extract as template (Effendi, 2010). Thermocycling conditions for the MPCR were as follows: initial incubation of 94ºC for 5 min followed by 40 cycles of denaturation at 94ºC for 1 min, annealing at 52ºC for 30 sec, elongation at 72ºC for 1 min and final extension of 72ºC for 10 min (Brenjchi *et al.*, 2011). Around 5 µl of the amplified product was mixed with 2 µl of loading solution and inserted into a 1.5% agarose gel. Electrophoresis was performed for 1 h with a constant voltage of 75 volts. After 1 h, PCR products were visualized under UV light. The primers used in the study is shown in Table 1.

Table 1. Primers used in the study. Adapted from Brenjchi *et al.*, 2011

Gene target	Primer sequence	Size (bp)
Escherichia coli O157:H7 (*flicH7*)	F: 5′- GCG CTG TCG AGT TCT ATC GAG-3′ R: 5′- CAA CGG TGA CTT TAT CGC CAT TCC-3′	625
stx2	F: 5′- CCA TGA CAA CGG ACA GCA GTT-3′ R: 5′- CCT GTC AAC TGA GCA CTT TG-3′	779

RESULTS AND DISCUSSION

Positive milk samples in BGBB were streaked in EMBA and positive *E. coli* samples, identified as colonies with metallic green appearance, were confirmed biochemically using Indol test. Of the 75 raw milk samples, around 26 were positive for *E. coli* (Table 2). The discovery of *E. coli* in milk may be caused by several factors, such as poor sanitation in the shed and unkempt cows (Hadiwiyoto, 1994). In addition, it was observed that the farm hands rarely wash their hands before and after milking. Khanal and Pandit (2013) stated that milk can harbor spread diseases, necessitating the person in charge of milking to maintain cleanliness. In this study, the incidence rate of contamination of *E. coli* was low (33% of the samples), but even so the presence of this bacteria in milk remains an important thing to note as *E. coli* has a low infective dose.

DNA extraction using DNAzol® Direct reagent and MPCR using primer pairs *flicH7* and *stx2* were performed. MPCR results showed that from 26 *E. coli* isolates, no DNA band was amplified using the *flicH7* primers; however, two samples were positive for *stx2* with amplicon length of 779 bp (Fig.). This may indicate that the raw milk samples analyzed were negative for *E. coli* serotype O157:H7. It is also possible that the absence of amplified bands may be because the sequences of *E. coli* O157:H7 in Indonesia are different from the sequence in other countries, especially in Iran where Brenjchi *et al.* (2011) conducted their study on *flicH7*. Sequence differences maybe due to environmental, climatic and seasonal factors that may cause bacteria to adapt to their environment, resulting to changes in the structure of genes in some bacterial components.

MPCR of *stx2* gene produced an amplified band of 779 bp in two of the 26 positive samples. This may suggest that the genes encoding for shiga toxin may not be derived from *E. coli* serotype O157:H7. Other enterohemorrhagic *E. coli* (EHEC) are examples of non- O157:H7 *E. coli* capable of producing shiga toxins (Martin and Beutin, 2011). According to Mainil and Daube (2005), all classes of EHEC isolated from animals, humans and foods can produce shiga toxin and lesions. Shiga toxin infections in humans from drinking milk can be avoided by consuming fully heated or pasteurized milk.

STEC are important foodborne pathogens. It contains *stx1* and *stx2* genes, encoding for cytotoxins that cause severe tissue damage, especially *stx2* which causes various human diseases ranging from diarrhea to hemorrhagic colitis (HC), thrombotic thrombocytopenia purpurea (TTP) and hemolytic uremic syndrome (HUS), with fatal consequences (Gyles, 2007; Petruzziello-Pellegrini and Marsden, 2012; Walker *et al.*, 2012). Ruminants, especially bovines, are the main reservoirs of STEC and human contamination are often associated with consumption of ground meat and direct contact with animals or their environment (Savoye *et al.,* 2011). STEC is highly pathogenic in humans in low infection doses and may cause illness brought by food through contaminated consumption of water or food (Dweik *et al.,* 2012). Cow's milk and other milk products like yogurt and cheese

Table 2. Bacterial isolation and Multiplex Polymerase Chair Reaction (MPCR) of shiga toxin-producing *Escherichia coli* (STEC) in raw milk samples from dairy cows in Surabaya, Indonesia.

	Name of farm	Milk samples	Positive *E. coli* (%)	Positive *stx2* gene (%)
1	Kl farm	20	9	0
2	Wn farm	20	8	2
3	Kb farm	20	3	0
4	Pg farm	15	6	0
	TOTAL	75	26 (34.7)	2 (2.7)

have been associated with disease caused by STEC (Martin and Beutin, 2011). Outbreaks of illness caused by milk associated with STEC, including pasteurized dairy products, have been reported worldwide (Seghal *et al.*, 2008).

Identification of the presence of pathogenic microorganisms in food is the gold standard for determining the source of food poisoning. In most clinical laboratories, identification procedures are mainly based on microbiological culture and biochemical tests. Some disadvantages of microbiological culture may include 1) inability to isolate target microorganisms due to presence of very low bacterial counts in the sample, 2) negative culture may also be due to residual presence of therapeutic antibiotics that can inhibit bacterial growth *in vitro*, 3) stressed bacteria, which may not grow directly on selective media unless allowed to recover (Riffon *et al.,* 2001).

Detection of STEC is labor-intensive and the total time required for strain characterization is usually 72 h. On the other hand, molecular methods are sensitive, specific and a quick approach to detection and characterization of microbiological contaminants in food. The molecular characterization of STEC is performed by means of multiplex PCR. MPCR showed that among all *E. coli* samples, 7.7% was contaminated with STEC. Environmental contamination, herd management and poor milking practices are important causes of milk degradation. It has been shown that food animals are important sources of STEC's entry into the food chain (Martin and Beutin, 2011). Its pathogenicity is associated with production of *stx1* and *stx2* as verocytotoxin (Hessain, *et al.*, 2015). Previous research has reported that *stx2* is the most important virulence factor and most hemolytic-uremic cases of syndrome in humans caused by STEC (Elhadidy and Mohammed, 2013). Further, Douellou *et al.* (2017) indicated that the virulence gene profile of dairy products and human STEC strains are similar. Nagachinta and Chen (2008) reported an association between STEC virulence factors and antimicrobial resistance of *E. coli* isolated from dairy cows.

The findings of this study on the

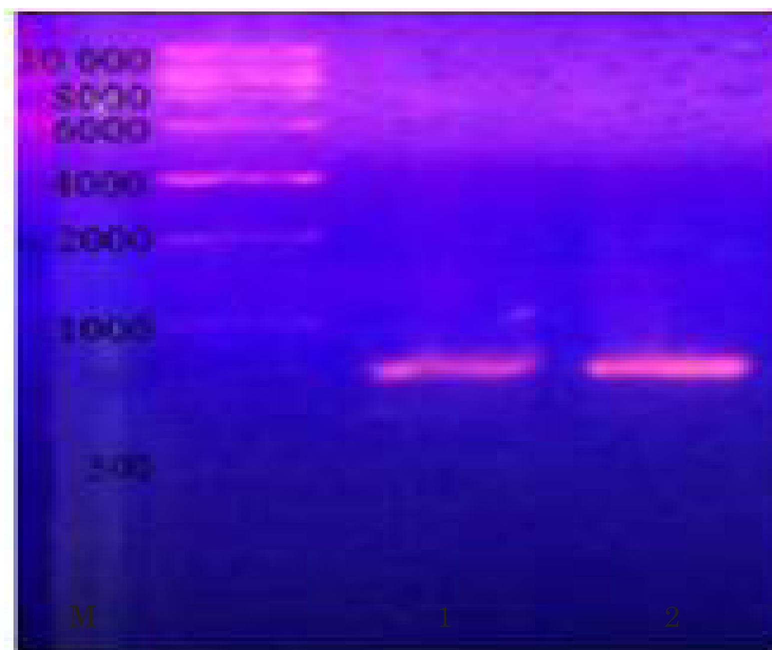

Fig. Multiplex Polymerase Chain Reaction of raw milk samples from dairy cows in Surabaya, Indonesia. M: molecular ladder, 1: sample 1, 2: sample 2.

contamination of raw milk are important and should be considered, since even one STEC colony in food samples can cause gastrointestinal or urogenital disruption (Gyles, 2007). Therefore, hygiene practices and strict management for dairy herds, and processing and storage of milk should be adopted to avoid undesirable illness due to contaminated milk and consumption of dairy products.

REFERENCES

Andriani. 2005. *Escherichia coli* O157:H7 as a cause of zoonotic disease. *Proceedings of the National Workshop on Zoonotic Disease* 173-177.

Bandyopadhyay S, Mahanti A, Samanta I, Dutta TK, Ghosh MK, Bera AK, Bandyopadhyay S and Bhattacharya D. 2011. Enterotoxigenic *Escherichia coli* (ETEC) from diarrhoeic lambs of Arunachal Pradesh, India. *Tropical Animal Health and Production* 43(3): 705-710.

Brenjchi M, Jamshidi A, Farzaneh N and Bassami MR. 2011. Identification of shiga toxin producing *Escherichia coli* O157:H7 in raw milk samples from dairy farms in Mashhad using multiplex PCR assay. *Iranian Journal of Veterinary Research* 12(2): 145-149.

Douellou T, Delannoy S, Ganet S, Fach P, Loukiadis E, Montel MC and Sergentet-Thevenot D. 2017. Molecular characterization of O157:H7, O26:H11 and O103:H2 shiga toxin-producing *Escherichia coli* isolated from dairy products. *International Journal of Food Microbiology* 253: 59-65.

Dweik M, Stringer RC, Dastider SG, Wu Y, Almasri M and Barizuddin S. 2012. Specific and targeted detection of viable *Escherichia coli* O157:H7 using a sensitive and reusable impedance biosensor with dose and time response studies. *Talanta* 94: 84-89.

Effendi MH. 2010. Species identification of coagulase positive staphylococci (CPS) by multiplex polymerase chain reaction (PCR). *Media Kedokteran Hewan* 26(2): 108-111.

Effendi MH, Harijani N and Budiarto. 2017. Profile antibiotics resistance on *Escherichia coli* isolated from raw milk in Surabaya dairy farms, Indonesia. *The Turkish Online Journal of Design, Art and Communication TOJDAC, Special Ed* 1340-1344.

Elhadidy M and Mohammed MA. 2013. Shiga toxin-producing *Escherichia coli* from raw milk cheese in Egypt: prevalence, molecular characterization and survival to stress conditions. *Lett. Appl. Microbiol.* 56(2): 120-127.

Gyles CL. 2007. Shiga toxin producing *Escherichia coli*: an overview. *Journal of Animal Science* 85(13): 45-62.

Hadiwiyoto S. 1994. *Theory and Procedure of Quality Testing of Milk and Processed Products* (2nd ed.). Yogyakarta, Indonesia: Liberty Press.

Hassan J, Parvej MS, Rahman MB, Khan MSR, Rahman MT, Kamal T and Nazir K. 2014. Prevalence and characterization of *Escherichia coli* from rectal swab of apparently healthy cattle in Mymensingh, Bangladesh. *Microbes and Health* 3: 12-14.

Hessain AM, Al-Arfaj AA, Zakri AM, El-Jakee JK, Al-Zogibi OG, Hemeg HA and Ibrahim IM. 2015. Molecular characterization of *Escherichia coli* O157:H7 recovered from meat and meat products relevant to human health in Riyadh, Saudi Arabia. *Saudi Joural of Biological Sci*ences 22(6): 725-729.

Hogan J and Smith KL. 2003. Coliform mastitis. *Veterinary Research* 34: 507-519.

Islam MA, Abdus SM, Boer E, Rijkelt RB, Marcel HZ, Kaisar AT and Heuvelink AE. 2008. Prevalence and genetic characterization of shiga toxin-producing *Escherichia coli* isolates from slaughtered animals in Bangladesh. *Applied and Environmental Microbiology* 74(17): 5414-5421.

Jiang X, Morgan J and Doyle MP. 2003. Thermal inactivation of *Escherichia coli* O157:H7 in cow manure compost. *Journal of Food Protection* 66(10): 1771-1777.

Khanal T and Pandit A. 2013. Assessment of sub-clinical mastitis and its associated risk factors in dairy livestock of Lamjung, Nepal. *International Journal of Infection and Microbiology* 2: 49-54.

Mainil JG and Daube G. 2005. Verotoxigenic *Escherichia coli* from animals, humans and foods: who's who? *Journal of Applied Microbiology* 98(6): 1332-1344.

Martin A and Beutin L. 2011. Characteristics of shiga toxin producing *Escherichia coli* from meat and milk products of different origins and association with food producing animals as main contamination sources. *International Journal of Food Microbiology* 146(1): 99-104.

Nagachinta S and Chen J. 2008. Transfer of class 1 integron-mediated antibiotic resistance genes from shiga toxin-producing *Escherichia coli* to a susceptible *Escherichia coli* K-12 strain in storm water and bovine feces. *Applied Environmental Microbiology* 74(16): 5063-5067.

Nakasone N, Tran HH, Nguyen MB, Higa N, Toma C, Song T, Ichinose Y and Iwangga M. 2005. Short report: isolation of *Escherichia coli* O157:H7 from fecal samples of cows in Vietnam. *The American Journal of Tropical*

Medicine and Hygiene 73(3): 586-587.

Petruzziello-Pellegrini TN and Marsden PA. 2012. Shiga toxin associated hemolytic uremic syndrome: advances in pathogenesis and therapeutics. *Current Opinion in Nephrology and Hypertension* 21(4): 433-440.

Riffon R, Sayasith K, Lhalil H, Dubreuil P, Drolet M and Lagace J. 2001. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *Journal of Clinical Microbiology* 39(7): 2584-2589.

Savoye F, Feng P, Rozand C, Bouvier M, Gleizal A and Thevenot D. 2011. Comparative evaluation of a phage protein ligand assay with real-time PCR and a reference method for the detection of *Escherichia coli* O157:H7 in raw ground beef and trimmings. *Journal of Food Protection* 74(1): 6-12.

Seghal R, Kumar Y and Kumar S. 2008. Prevalence and geographical distribution of *Escherichia coli* O157 in India: a 10 year survey. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 102(4): 380-383.

Stephan R, Schumacher S, Corti S, Krause G, Danuser J and Beutin L. 2008. Prevalence and characteristics of shiga toxin-producing *Escherichia coli* in Swiss raw milk cheeses collected at producer level. *Journal of Dairy Science* 91(7): 2561-2565.

Suwito W. 2010. Bacteria that often contaminate milk: detection, pathogenesis, epidemiology, and how to control it. *Jurnal Penelitian and Pengembangan Pertanian* 29(3): 96-100.

Walker CL, Applegate JA and Black RE. 2012. Haemolyticuraemic syndrome as a sequel of diarrhoeal disease. *Journal of Health, Population and Nutrition* 30(3): 257-261.

RESEARCH NOTE

TETRACYCLINE RESISTANCE GENE IN *Streptococcus agalactiae* ISOLATED FROM BOVINE SUBCLINICAL MASTITIS IN SURABAYA, INDONESIA

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ABSTRACT

The aim of this research was to isolate, identify and determine tetO resistance genes in tetracycline-resistant *Streptococcus agalactiae* isolated from cows with subclinical mastitis in Surabaya and surrounding areas of Indonesia. Milk samples from cows with subclinical mastitis in six dairy farms were collected. *S. agalactiae* was isolated and antibiotic resistance was determined. Results showed that out of 173 samples analyzed, 131 (75.7%) were positive for California Mastitis Test. *S. agalactiae* was isolated in 36 out of the 131 CMT-positive samples. Antibiotic sensitivity test revealed that out of 36 *S. agalactiae* samples, nine were resistant to tetracycline. PCR analysis showed that six of the nine tetracycline resistant *S. agalactiae* isolates were positive for the *tetO* resistance genes.

Key words: *Streptococcus agalactiae,* subclinical mastitis, *tetO* gene, tetracycline resistance

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INTRODUCTION

Streptococcus agalactiae is an important cause of chronic, contagious bovine mastitis. It also causes mastitis and invasive disease in camels and is an occasional cause of disease in dogs, cats, fish and hamsters. Its presence is frequently associated with high somatic cell counts in milk and decreased milk yield (Jain *et al.,* 2012).

There are two kinds of mastitis: clinical mastitis with clearly defined clinical signs and subclinical mastitis with unobservable clinical signs (Hashemi *et al.,* 2011). Subclinical mastitis is the most dominant form in Indonesia (Effendi and Harijani, 2017) and can be found in Bogor (76%), Boyolali (91%) and Malang (81%). Differences in incidence rate of subclinical mastitis by area are also observed, in which Yogyakarta has 72%; Central Java, 65%; and East Java, 44.46% (Sudarwanto and Sudarnika, 2008; Wahyuni, 2005).

S. agalactiae and *Staphylococcus aureus* are common causes of bovine mastitis. Although there are plenty of research on *Staphylococcus* in Indonesia, research on *S. agalactiae* is limited. Therefore, it will be useful to do research on this pathogen for guidance on the prevention and control of mastitis and also for public health awareness. According to Dogan *et al.* (2005) and Songer and Post (2005), *S. agalactiae* can cause various diseases to humans, such as bacterial sepsis, pneumonia, meningitis, Scarlet fever and tonsillitis (Duarte *et al.,* 2005).

S. agalactiae often causes subclinical mastitis in dairy cattle causing economic loss for the industry (Alemu *et al.*, 2014). Dairy farmers ranked mastitis as a major disease problem in their farms (Carvalho-Castroa *et*

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al., 2017). Veterinarians are often asked to provide information for herd level control and eradication of *S. agalactiae*. Farmers are often involved with veterinarians in the treatment using antibiotics, especially tetracyclines, to solve mastitis problem (Jain *et al*., 2012).

Tetracycline is one of the most commonly used antibiotic in many developing countries, both in human and veterinary medicine. The main reasons are its relatively low cost and availability (Zibandeh *et al*., 2016). This class of antibiotics is still used in developed countries for prophylactic and therapeutic purposes. The widespread use of tetracycline in dairy farming could result in horizontal transfer of resistance from bovine to humans as well as to the environment. Treatment with intramammary infusion of antibiotics is the main approach to deal with mastitis, and a number of *in vivo* and *in vitro* trials to assess the antibiotic sensitivity/resistant pattern have been documented. However, there are few reports focusing on the genes involved in resistance especially for *S. agalactiae* isolates of bovine origin. The present study aims to identify tetracyline resistant gene in *S. agalactiae* isolated from subclinical mastitis cases.

MATERIALS AND METHODS

Sample collection

Milk samples were collected from six dairy farms in Surabaya, namely Kaliwaron, Sutorejo, Wonocolo, Sepanjang, Taman and Wonoayu (Table 1). The six farms were visited during the afternoon milking. Complete herd size (including calves and young stock) varied between 14-83 animals and number of lactating cows (only counting the animals being milked at the time of the visit) varied between 10-65 cows. In total, 173 animals were examined. Before sampling, the teats were scrubbed with cotton soaked in 70% ethanol and the first squirt of milk was discarded. Approximately, 10 ml of milk was collected from each teat and samples from one cow were pooled together as one sample (Effendi and Harijani, 2017). Milk samples were placed in sterile tubes and stored in ice box during transport. A total of 173 milk samples were collected from individual cows.

California mastitis test

Cases of subclinical mastitis based on California mastitis test (CMT) were investigated. CMT is a simple indicator of the somatic cell count in milk. Positive test reactions were graded by visually - Grades 0, +1, +2 and +3). Grade +1 shows formation of solid gels; Grade +2 shows formation of solid thick gels at the *paddle* center; and Grade +3 shows large number of solid gels with convex surface (Björk, 2013).

Identification of *Streptococcus agalactiae*

Milk samples were streaked in nutrient agar (NA) (E. Merck, Darmstadt, Germany) and incubated for 24 h at 37°C. The isolates were subcultured in blood agar (BA) (E. Merck, Darmstadt, Germany) to identify the *Streptococcus* with characteristic ά-hemolysis, ß-hemolysis or without hemolysis/γ-hemolysis. Suspected *Streptococcus* spp. was characterized using gram staining and catalase test. *Streptococcus* colonies with ß-hemolysis

Table 1. Microbiological analysis and AST results of milk samples from Surabaya, Indonesia.

Name of farm	Number of population	Number of samples	CMT-Positive	*S. agalactiae* positive	Tetracycicline resistant
Kl farm	35	20	15	7	1
Wn farm	83	50	42	10	2
St farm	14	8	6	4	none
Sp farm	47	30	21	6	3
Tm farm	75	35	24	4	1
Wy farm	62	30	23	5	2
TOTAL	316	173	131	36	9

were characterized using Christie-Atkins-Munch-Peterson test (CAMP) to identify *S. agalactiae* strains (Ahmadi *et al.,* 2009).

Antibiotic sensitivity test

To detect for antibiotic resistance, the disc diffusion method, as described by Lopez-Lazaro *et al.,* (2000) was employed and the interpretation was made according to the zone size interpretation chart provided by the disc manufacturer.

Polymerase chain reaction

DNA extraction was carried out as described by Rato *et al.* (2013) with minor modifications: doubling the time of centrifugation, the amount of enzymes and addition of a final step for DNA precipitation by ethanol. Briefly, 1 ml of each sample was transferred to a microtube and centrifuged at 14,000 rpm for 4 min. The supernatant was discarded, and the pellet was re-suspended and washed 2-3 times with Tris-EDTA buffer (Tris-HCL 10 mM, EDTA 1 ml, pH 8.8) until a clear solution was obtained. The pellet was washed with PCR buffer (Buffer 10X: Tris- HCl 100 mM, KCl 500 mM, pH 8.8) and finally resuspended in 100 µl of PCR buffer. Thereafter, lysozyme (Merck, Germany) was added to each sample at a concentration of 2 mg/ml, and the sample was incubated for 20 min at room temperature. After this, proteinase K (Fermentas, Germany) was added at a concentration of 400 µg/ml and the sample was incubated at 56°C for 1 h. The sample was then boiled for 15 min and centrifuged at 14,000 rpm for 45 sec.

Approximately, 5 µl of DNA extract was used as template for the PCR amplification of the *tetO* gene fragment. In brief, 20 µl of PCR reaction consisted of 12.5 µl master mix, 0.5 µl distilled water, 1 µl of forward and reverse primers (Table 2) and template DNA. Thermocycling conditions were as follows: prewarming at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 58°C for 1 min and 72°C for 1 min 30 sec (Jain *et al.,* 2012). Electrophoresis was performed at 110V for 30 min. PCR products were stained by ethidium bromide and observed under ultraviolet light.

RESULTS AND DISCUSSION

Analysis of milk samples showed that 131 out of the 173 samples (75.7%) were positive for CMT. This result showed was similar with other reports showing high evidence rate of subclinical mastitis in East Java area. Previous reports showed that the prevalence rate of mastitis in dairy farms around East Java was at 80-86%; Nongkojajar at 82.7%; Batu at 83.1%; Surabaya at 86.4%; and Grati at 79.5% (Effendi, 2008). This study showed that the majority of subclinical cases of mastitis were due to contagious pathogens such as *S. aureus* and *S. agalactiae.* This might be related to poor milking and mastitis control practice seen in the studied farms. In the absence of hygienic milking practice, pathogens from either infected cow or dirty hands (from milking) can easily spread.

Table 2. Primers for *tetO* gene for milk samples from cows with subclinical mastitis.

Gene	Primer sequence	Position	Size amplification (bp)
tetO	F: 5′-GCGTCAAAGGGGAATCACTATCC-3′ R: 5′-CGGCGGGGTTGGCAAATA-3′	146-169 1851-1868	1723 bp

Source: Jain *et al.* (2012).

Bacterial isolation was performed on CMT positive samples using morphology, gram staining and CAMP tests (Fig. 1). Thirty-six samples were positive for *S. agalactiae.* Furthermore, antibiotic sensitivity test showed that nine isolates were resistant to

tetracyclines (30 ug).

Tetracycline is a family of broad-spectrum antibiotics often used in livestock production. The first generation tetracyclines, such as tetracycline, chlortetracycline and oxytetracycline, have been widely used as a growth promoter for decades and the second generation, such as minocycline and doxycycline, is commonly used both in prophylaxis and therapeutics in humans and animals (Eliopoulos and Roberts, 2003). Tetracycline is used for all animal food production species, mainly because of its wide-spectrum activities, price and availability. However, extensive use of tetracyclines can lead to the emergence of resistant bacteria (Chopra and Roberts, 2001). The extended use of tetracycline may result to selection pressure and, ultimately, resistance.

PCR results showed that six out of the nine tetracycline resistant *S. agalactiae* samples were positive for *tetO* genes, with PCR bands of 1723 bp (Fig. 2). Out of the nine tetracycline resistant isolates, six isolates were found positive for tetracycline resistance gene (*tetO*); three isolates were negative (Table 3).

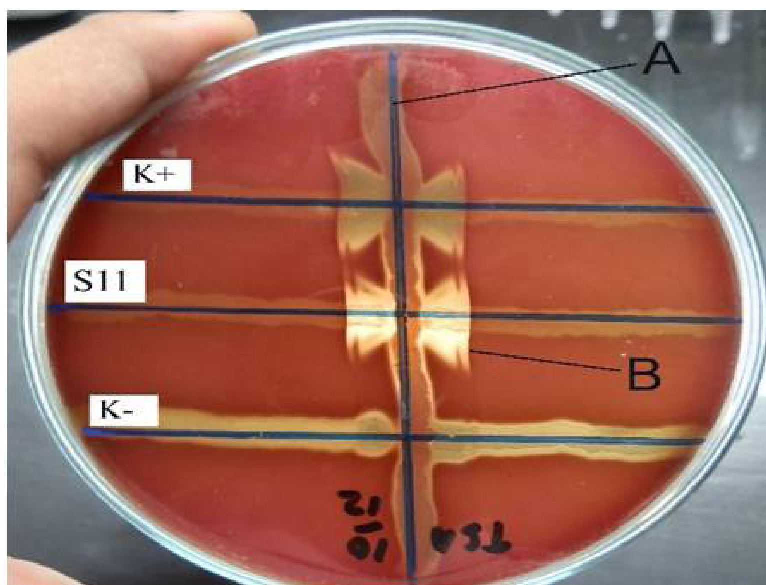

Fig 1. Christie-Atkins- Munch-Peterson (CAMP) test result of milk sample from cow with subclinical mastitis. A: *Stapylococcus aureus* bacteria; B: arrow marks that show CAMP test results; K+: Positive control, *Streptococcus agalactiae*; S11: sample; K-: negative control, *Streptococcus pyogenes*.

Streptococcus tetracycline resistance genes were *tetL, tetM, tetO, tetQ* and *tetT*. The genes of *tetO* and *tetM* were identified as the dominant tetracycline resistance encoding gene, where *tetM* is found in *S. agalactiae* from human isolates and *tetO* gene in *S. agalactiae* from dairy isolates (Dogan *et al.,* 2005). Research by Duarte *et al.* (2005) showed that the major tetracycline gene is the *tetO* gene from 27 of 38 milk samples (71%).

The mechanism of action of tetracyclines has been reviewed by Velhner and Milanov (2015). Mainly, tetracyclines inhibit reversible protein synthesis of bacteria by binding to the ribosomal complex, preventing the aminoacyl-tRNA association with bacterial ribosomes. This results to weakened interaction of the ribosome-tRNA, thus halting protein synthesis.

Bryan *et al.* (2004) reported that environment, human and animal exposure to tetracyclines, as well as to other antibiotics may contribute to the development and spread of antibiotic resistance through horizontal gene transfer. *S. agalactiae* infections in both humans and bovines are treated by

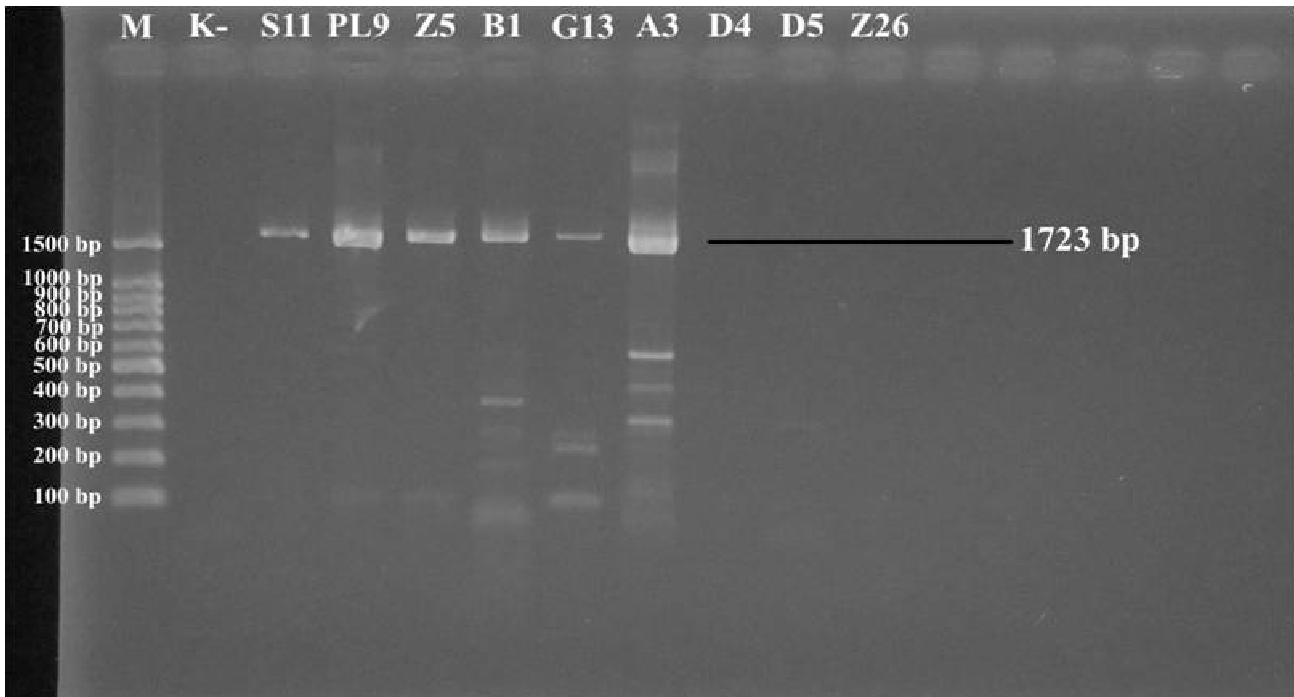

Fig. 2. PCR analysis of *tetO* gene in tetracycline resistant *S. agalactiae* from milk sample in cow with subclinical mastitis; *tetO* gene is indicated by the DNA band at 1723 bp.

Table 3. Determination of *tetO* resistance genes in tetracycline resistant *S. agalactiae* from milk sample in cow with subclinical mastitis.

Name of farm	Tetracycline resistant *S. agalactiae*	*tetO* gene
Kl farm	1	1
Wn farm	2	1
St farm	none	none
Sp farm	3	2
Tm farm	1	none
Wy farm	2	2
TOTAL	9	6

administration of antibiotics (Jake *et al.,* 2013). Extensive use of antibiotics in medicine and animal husbandry results to increased antibiotic resistance among bacterial populations (Gao *et al.,* 2012). Several studies have suggested that antimicrobial use in animals causes the development of antibiotic resistance among pathogens in humans (Dogan *et al.,* 2005). Therefore, an effective information campaign is needed to create awareness on the spread of antimicrobial resistance and the requirements on proper hygiene and adoption of other preventive measures by rural farmers to reduce losses due to subclinical mastitis.

REFERENCES

Ahmadi M, Razavi Rohani SM and Ayremlou N. 2009. Evaluation of *Streptococcus agalactiae* detection by PCR in milk and its comparison to other microbiological methods. *Iranian Journal of Microbiology* 1(4): 28-31.

Alemu G, Almaw G and Abera M. 2014. Incidence rate of *Staphylococcus aureus* and *Streptococcus agalactiae* in subclinical mastitis at smallholder dairy cattle farms in Hawassa, Ethiopia. *African Journal of*

Microbiology Research 8(3): 252-256.

Björk S. 2013. Clinical and subclinical mastitis in dairy cattle in Kampala, Uganda. Uppsala, SLU pp 1-42.

Bryan A, Shapir N and Sadowsky MJ. 2004. Frequency and distribution of tetracycline resistance genes in genetically diverse, nonselected, and nonclinical *Escherichia coli* strains isolated from diverse human and animal sources. *Applied and Environmental Microbiology* 70: 2503-2507.

Carvalho-Castro GA, Silva JR, Paiva LV, Custódio DAC, Moreira RO, Mian GF, Prado IA, Chalfun-Junior A and Costa GM. 2017. Molecular epidemiology of *Streptococcus agalactiae* isolated from mastitis in Brazilian dairy herds. *Brazilian Journal of Microbiology* 48: 551-559.

Chopra I and Roberts M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* 65: 232-260.

Dogan BYH, Schukken YH, Santisteban C and Boor KJ. 2005. Distribution of serotypes and antimicrobial resistance genes among *Streptococcus agalactiae* isolates from bovine and human host. *Journal of Clinical Microbiology* 43(12): 5899-5906.

Duarte RS, Bellei BC, Miranda OP, Brito MA and Teixeira LM. 2005. Distribution of antimicrobial resistance and virulence-related genes among Brazilian group B *Streptococci* recovered from bovine and human sources. *Antimicrobial Agents Chemotherapy* 49(1): 97-103.

Effendi MH. 2008. Bovine mastitis prevalence figures from several dairy farms in East Java**.** *Veterinaria Medika* 1: 1-6.

Effendi MH and Harijani N. 2017. Cases of methicillin-resistant *Staphylococcus aureus* (MRSA) from raw milk in East Java, Indonesia. *Global Veterinaria* 19(1): 500-503.

Eliopoulos GM and Roberts MC. 2003. Tetracycline therapy: update. *Clinical Infectious Diseases* 36: 462-467.

El-Jakee J, Hableel HS, Kandil M, Hassan OF, Eman AK and Marouf SA. 2013. Antibiotic resistance pattern of *Streptococcus agalactiae* isolated from mastitic cows and ewes in Egypt. *Global Veterinaria* 10(3): 264-270.

Gao J, Fu-Qing Y, Li-Ping L, Jian-Zhong H, Rong-Guang H, Han-Qi Z, Shu-Mei L, Jing-Liang S and Bo H. 2012. Antibiotic resistance of *Streptococcus agalactiae* from cows with mastitis. *The Veterinary Journal* 194(3): 423-424.

Hashemi M, Kafi M and Safdarian M. 2011. The prevalence of clinical and subclinical mastitis in dairy cows in the central region of Fars Province, South of Iran. *Iranian Journal of Veterinary Research* 12(3): 236- 241.

Jain B, Tewari A, Bhandari BB and Jhala MK. 2012. Antibiotic resistance and virulence genes in *Streptococcus agalactiae* isolated from cases of bovine subclinical mastitis. *Veterinarski Arhiv* 82(5): 423-432.

Lopez-Lazaro JM, Monnet L, Yagüe A, Burgos A, Gonzalo A, Campillos N and Saez M. 2000. Modelling and forecasting antimicrobial resistance and its dynamic relationship to antimicrobial use: a time series analysis. *International Journal of Antimicrobial Agents* 14(1): 21-31.

Nam HM, Lim SK, Kang HM, Kim JM, Moon JS, Jang KC, Joo YS, Kang MI and Jung SC. 2009. Antimicrobial resistance of *Streptococci* isolated from mastitic bovine milk samples in Korea. *Journal of Veterinary Diagnostic Investigation* 21(5): 698-701.

Rato MG, Ricardo B, Carlos F, Lina MC, Cristina LV and Ilda SS. 2013. Antimicrobial resistance and molecular epidemiology of *Streptococci* from bovine mastitis. *Veterinary Microbiology* 161(3-4): 286-294.

Songer JG and Post KW. 2005. *Veterinary Microbiology Bacterial and Fungal Agents of Animal Disease*. London: Elsevier Saunders.

Sudarwanto M and Sudarnika E. 2008. Relationship between milk pH and somatic cell counts as parameters of subclinical mastitis. *Media Peternakan* 31(2): 107-113.

Velhner M and Milanov D. 2015. Resistance to tetracycline in *Escherichia coli* and *Staphylococcus aureus*: brief overview on mechanisms of resistance and epidemiology. *Archives of Veterinary Medicine* 8(1): 27-36.

Wahyuni AETH, Wibawan IWT and Wibowo MH. 2005. Characterization of hemaglutinin in *Streptococcus agalactiae* and *Staphylococcus aureus* causes subclinical mastitis in dairy cattle. *Journal Sains Veteriner* 23(2): 70-77.

Zibandeh S, Sharifiyazdi H, Asasi K and Abdi-Hachesoo B. 2016. Investigation of tetracycline resistance genes in *Escherichia coli* isolates from broiler chickens during a rearing period in Iran. *Veterinarski Arhiv* 86(4): 565-572.

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