

The PHILIPPINE JOURNAL OF

Veterinary Medicine

Volume 55

Special Issue

December 2018

**Published by the College of Veterinary Medicine
University of the Philippines Los Baños**

ISSN 0031-7705



The 2nd Veterinary Medicine
International Conference
Surabaya, Indonesia
4-5 July 2018

The Philippine Journal of Veterinary Medicine

Volume 55

Special Issue

December 2018

The Philippine Journal of Veterinary Medicine is a peer-reviewed international journal of basic and applied research in veterinary medicine and science. It is published semi-annually, for the periods January-June and July-December each year, by the College of Veterinary Medicine, University of the Philippines Los Baños. All articles are subjected to double-blind review.

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This journal is Abstracted/Indexed by: SCOPUS, Biological Abstracts, Focus on: Veterinary Science & Medicine, Zoological Records, CAB Abstracts, Index Veterinarius, Veterinary Bulletin, Parasitology Database, Helminthological Abstracts, Protozoological Abstracts, Review of Medical and Veterinary Entomology, EBSCO, ASEAN Citation Index, Prescopus Russia, *i-journals* (www.ijournals.my), *i-focus* (www.ifocus.my), *i-future* (www.ifocus.my), Philippine E-Journals (<https://ejournals.ph>) and UPLB Journals Online (<http://journals.uplb.edu.ph/index.php/PJVM>).

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

Therigenology

- 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

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

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

Philipp. J. Vet. Med., 55(SI): 109-114, 2018

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 BGGB media (E. Merck, Darmstadt, Germany) and incubated at 37°C for 18-24 h. Positive samples on BGGB, 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 37°C for 18-24 h. Confirmed *E. coli* isolates were subsequently cultured in 5 ml lactose broth and incubated at 37°C 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 37°C 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 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 30 sec, elongation at 72°C for 1 min and final extension of 72°C 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)
<i>Escherichia coli</i> O157:H7 (<i>flicH7</i>)	F: 5'- GCG CTG TCG AGT TCT ATC GAG-3'	625
	R: 5'- CAA CGG TGA CTT TAT CGC CAT TCC-3'	
<i>stx2</i>	F: 5'- CCA TGA CAA CGG ACA GCA GTT-3'	779
	R: 5'- CCT GTC AAC TGA GCA CTT TG-3'	

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 purpura (TTP) and hemolytic uremic syndrome (HUS), with fatal consequences (Gyles, 2007; Petruzzello-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

Table 2. Bacterial isolation and Multiplex Polymerase Chain 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 <i>E. coli</i> (%)	Positive <i>stx2</i> 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)

and other milk products like yogurt and cheese 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.

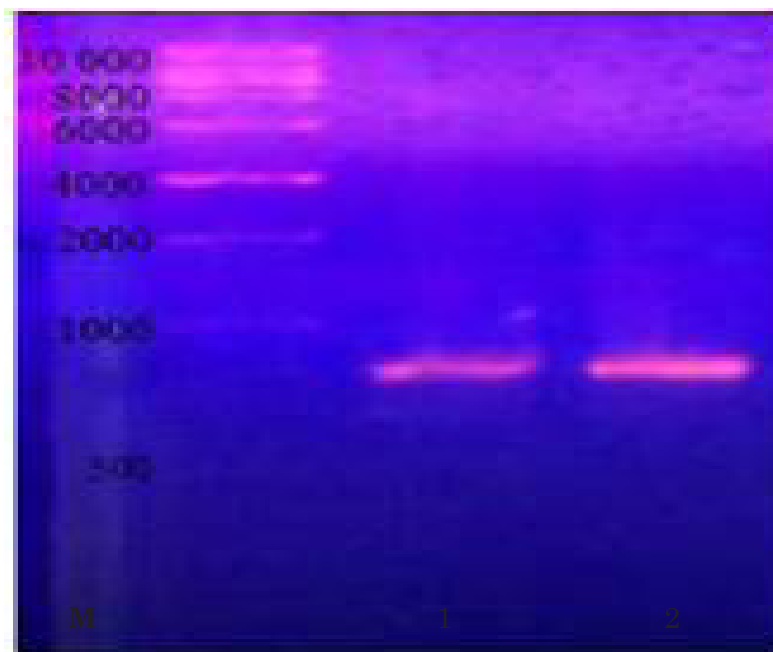


Fig. Multiplex Polymerase Chain Reaction of raw milk samples from dairy cows in Surabaya, Indonesia. M: molecular ladder, 1: sample 1, 2: sample 2.

The findings of this study on the 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.

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