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

by Mustofa Helmi

Submission date: 31-Dec-2019 10:59AM (UTC+0800)
Submission ID: 1238821708
File name: 9._Identification_of_Shiga_Toxin.....pdf (350.55K)
Word count: 2987
Character count: 15800

RESEARCH NOTE

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

Mustofa Helmi Effendi*[1], Nenny Harijani[1], Sheila Marty Yanestria[2] and Poedji Hastutiek[3]

[1]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Airlangga; [2]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Wijaya Kusuma University; [3]Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia

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,

*FOR CORRESPONDENCE:
(email: mheffendi@yahoo.com)

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 37°C 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 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)
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

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)

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.

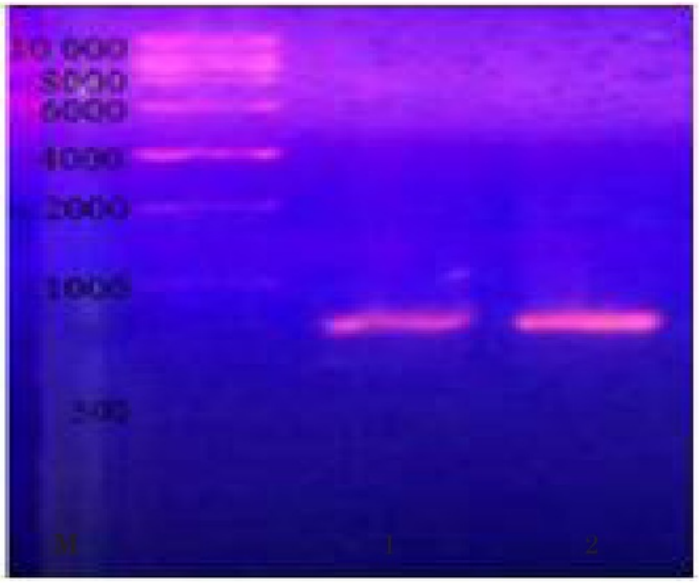

Fig. Multiplex Polymerase Chain Reaction of raw milk samples from dairy cows in Surabaya, Indonesia. M: molecular ladder, 1: sample 1, 2: sample 2.

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.

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.

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

ORIGINALITY REPORT

20%	15%	15%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.veterinaryworld.org Internet Source	4%
2	www.aun.edu.eg Internet Source	1%
3	www.myfoodresearch.com Internet Source	1%
4	www.im.microbios.org Internet Source	1%
5	Ombarak, Rabee A., Atsushi Hinenoya, Sharda Prasad Awasthi, Atsushi Iguchi, Ayaka Shima, Abdel-Rahman M. Elbagory, and Shinji Yamasaki. "Prevalence and pathogenic potential of Escherichia coli isolates from raw milk and raw milk cheese in Egypt", International Journal of Food Microbiology, 2016. Publication	1%
6	baadalsg.inflibnet.ac.in Internet Source	1%

7	Ashraf M. Ahmed, Tadashi Shimamoto. "Isolation and molecular characterization of Salmonella enterica, Escherichia coli O157:H7 and Shigella spp. from meat and dairy products in Egypt", International Journal of Food Microbiology, 2014 Publication	1%
8	Claudia Cafarchia. "Genetic variants of Malassezia pachydermatis from canine skin: body distribution and phospholipase activity", FEMS Yeast Research, 5/2008 Publication	1%
9	M. Baxendale, K. G. Lim, G. A. J. Amaratunga. "Thermoelectric power of aligned and randomly oriented carbon nanotubes", Physical Review B, 2000 Publication	1%
10	Hu, Jia, and Mei-Jun Zhu. "Defects in polynucleotide phosphorylase impairs virulence in Escherichia coli O157:H7", Frontiers in Microbiology, 2015. Publication	1%
11	Ashgan M. Hessain, Abdullah A. Al-Arfaj, Adel M. Zakri, Jakeen K. El-Jakee et al. "Molecular characterization of Escherichia coli O157:H7 recovered from meat and meat products relevant to human health in Riyadh, Saudi	1%

	Arabia", Saudi Journal of Biological Sciences, 2015 Publication	
12	slidelegend.com Internet Source	1%
13	academic.oup.com Internet Source	1%
14	Vo Van Giau, Thuy Trang Nguyen, Thi Kim Oanh Nguyen, Thi Thuy Hang Le, Tien Dung Nguyen. "A novel multiplex PCR method for the detection of virulence-associated genes of Escherichia coli O157:H7 in food", 3 Biotech, 2015 Publication	1%
15	agrifoodscience.com Internet Source	<1%
16	pubs.sciepub.com Internet Source	<1%
17	ir.amu.ac.in Internet Source	<1%
18	repositorio.unesp.br Internet Source	<1%
19	epdf.tips Internet Source	<1%
20	www.sdiarticle2.org	

	Internet Source	<1%
21	etd.aau.edu.et Internet Source	<1%
22	Cansu Onlen, Nizami Duran, Suphi Bayraktar, Emrah Ay, Burçin Ozer. "The frequency of shiga-like toxin (stx1 and stx2) and EHEC-hlyA in food by multiplex PCR", Revista Romana de Medicina de Laborator, 2017 Publication	<1%
23	Belgin Sarimehmetoglu, Mihriban Hatun Aksoy, Naim Deniz Ayaz, Yildiz Ayaz, Ozlem Kuplulu, Yusuf Ziya Kaplan. "Detection of Escherichia coli O157:H7 in ground beef using immunomagnetic separation and multiplex PCR", Food Control, 2009 Publication	<1%
24	Radhika Desikan, Steven J Neill, John T Hancock. "Hydrogen peroxide–induced gene expression in Arabidopsis thaliana", Free Radical Biology and Medicine, 2000 Publication	<1%
25	I. Saleh, O. Zouhairi, N. Alwan, A. Hawi, E. Barbour, S. Harakeh. " Antimicrobial resistance and pathogenicity of isolated from common dairy products in the Lebanon ", Annals of Tropical Medicine & Parasitology, 2013	<1%

Publication

26	Jamshidi A. "Quantification of Escherichia coli O157:H7 in milk by most probable number – polymerase chain reaction (MPN-PCR) method", African Journal of Microbiology Research, 2011 Publication	<1%

Exclude quotes	Off	Exclude matches	Off
Exclude bibliography	On		