MONITORING OF Eschericia. coli O157 FROM RAW COW'S MILK IN THE STORAGE TANK IN SLEMAN DISTRICT, YOGYAKARTA

Marcella Indah Kristanti, Tri Yahya Budiarso
Faculty of Biotechnology, Duta Wacana Christian University (DWCU) Yogyakarta, Indonesia
Corresponding author: Tel. : +6281392580765; Fax: +62274513235
Email: yahya@ukdw.ac.id

Abstract

Escherichia coli O157 is a member of Enterobacteriaceae which has somatic antigen O157. E. *coli* O157 is associated with life threatening diseases such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Raw milk is considered a high risk food as it is highly nutritious and serves as an ideal medium for bacterial growth. The aim of this study was to monitor *E. coli* O157 contamination in the storage tank before distribution in Sleman district, Yogyakarta. Total of 30 raw milk samples were collected from the storage tank in Sleman district. Tryptic Soy Broth (TSB) media added with novobiocin was used as enrichment medium, while Chromocult Coliform Agar (CCA) and Chromagar O157 medium for screening test. Additional analysis including serologic and molecular test of isolates obtained. Based on the screening result, 11,428 colonies were considered as *E. coli* O157 suspect that produced red colour in CCA medium. Further screening employing Chromagar O157 medium resulted in 3 potential colonies which produce mauve colour. These colonies were later tested with Latex Test O157 for serological reason, showing that none were *E. coli* O157. Molecular analysis with primer pairs for detection of *Stx1* and *Stx2* genes confirm that none of the suspected strains have genes that encoded the toxin, *Stx1* and *Stx2*. These results showed that the presence of STEC (*Shiga toxin E. coli*) hasn't found in the tested samples of raw cow's milk.

Keywords: *E. coli O157, raw cow's milk, storage tank, PCR*

1. Introduction

Escherichia coli O157 is a pathogenic bacteria, member of Enterobacteriaceae which is an important cause of severe diseases. *Escherichia coli* O157 can produced shiga toxin (STEC) and considered as verocytotoxin producing *E. coli* (VTEC) [6,20]. STEC was produced by *E. coli* O157 causes several diseaseas such as: stomach cramps, bloody diarrhoea (hemorrhagic colitis), fever, vomitting, Hemolytic Uremic Syndrome (HUS), and even death [6,21]. The effect of STEC causes bloody diarrhoea first recognized in 1982 in USA [14,11] and also causes HUS [11]. Cases from STEC have been reported in many countries until 2013 [3,8,4,5]. The biggest outbreak of STEC occurred in Japan in 1996 with fatality of up to 5,727 patients [13].

Escherichia coli O157 can found in some raw milk and milk product such as: cheese from raw milk, pasteurized milk, and yoghurt, because *E. coli* O157 live in intestine and can adhere in cow's hide include thigh and udder when the cow discard the feces then contaminate the product [3,20,21].

Several methods and biochemical screening have been reported for identifying *E. coli* O157:H7 including the toxin (STEC) they produced. For identifying the presence or absence of STEC *E. coli* O157:H7, PCR (polymerase chain reaction) technique must be employed for achieving this purpose. PCR can differentiate among types or variants of SLT genes, *i.e.*, *Stx1* and *Stx2*.

The aim of this study is molecularly identify the presence or absence of shiga toxin *E. coli* O157 from storage tank in Sleman district, Yogyakarta, employing primer pairs targetting *Stx1* and *Stx2* genes.

2. Materials and Methods

2.1 Sample collection

A total of 30 raw milk samples were collected daily from storage tank in Sleman district, Yogyakarta between February and April 2013. Samples were stored in the refrigerator before further analysis in the Laboratory of Microbiology Duta Wacana Christian University.

2.2. Isolation and Screening *E. coli* O157

A 50 ml of raw milk sample was centrifuged (Hanna HI 8314) at 4000 rpm for 10 min. The pellets were suspended in TSB that supplemented with novobiocin (final concentration, 20mg/ml; Sigma Chemical Co., St. Louis, Mo.), and the preparation was incubated at 37°C for 18-20 hours, with shaking 150 rpm. Next, samples were serially diluted in 0,1 % Bacto Peptone(Difco), and 1 ml

portions of the 10^{-7} and 10^{-10} dilutions were spread plated onto duplicate plates on Chromocult Coliform Agar. In the follow-up study, colonies that appeared salmon to red on the CCA medium were transferred on the Chromagar O157 for screening *E. coli* O157. Colonies of *E. coli* O157 growing on BBL CHROMagar O157 (CHROM) (Becton Dickinson) produce a mauve color due to chromogenic substrates in the medium. Mauve Colonies candidates O157 per sample were tested for the O157 antigen by using O157 latex agglutination test (Oxoid, Basingstoke, England). Colonies that agglutinated were streaked onto CCA ,incubated overnight, and retested for the O157 antigen. Agglutination-positive isolates were then transferred to brain heart infusion (Difco) agar slants until biochemical and serological tests were conducted. Colonies were confirmed biochemically as *E. coli* by using an API 20E biochemical test strip (bioMe´rieux Vitek, Inc.,Hazelwood,Mo.)

2.3 DNA extraction

Sucpected colonies grown on selective media were randomly picked, and transferred to Brain Heart Infusion broth, grown at 37°C overnight. Following the incubation, it is then centrifuged at 6,500 rpm for 3 minute. The supernatant was discarded and DNA was isolated from the pellet by standart method [15].

2.4 PCR assay

The primer pairs targetting *Stx1* and *Stx2* genes and its PCR condition were selected based on the available publications [7,18,10,9]. The primers used in this study were: Stx1–F: ACA CTG GAT GAT CTC AGT GG, Stx1–R: CTG AAT CCC CCT CCA TTA TG, Stx2–F: CCA TGA CAA CGG ACA GCA GTT and Stx2–R: CCT GTC AAC TGA GCA CTT TG.

Amplification of bacterial DNA was performed in 50 µl reaction mixture containing 1 µl (10µM) each of forward and reverse primer, 1µl DNA, 22 µl PCR mix (Fermentas), and distilled water. The amplification was done by using thermal cycler (PEQ-STAR) with the condition: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94° C for 1 min, 56°C for 30 s and 72°C for 1 min. The final cycle was followed by incubation for 10 min at 72°C. PCR products were separated on 1.2% agarose containing 0.1% Sybr® Safe DNA gel stain (Life technologies) for detection of the resulting fragments. It is then visualized on UV illuminator and documented by Gel Imager (Protein simple).

3. Results

Sample of 30 raw milks were analyzed on the CCA medium, resulted 11.428 colonies that were negative sorbitol. Colonies candidate were screening in Chromagar O157 to suspect for *E. coli* O157. Based on the screening found 3 isolates were considered as potential O157. Further analysis done by confirming serological Latex Test O157. The result showed there is no agglutination from all the isolate tested (Figure 1). Attempt to identify the present of toxin genes was done by conducting PCR employing primer pairs targeting *Stx1* and *Stx2* genes. The result is shown in Figure 2.

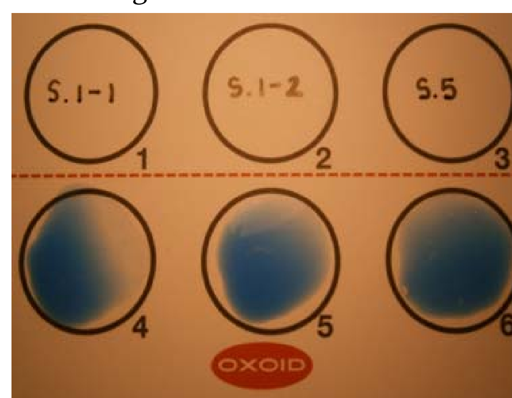

Figure 1. Latex Test result from 3 isolates shown that no agglutination occured. Sample 1 (left), sample 2 (middle), and sample 3 (right)

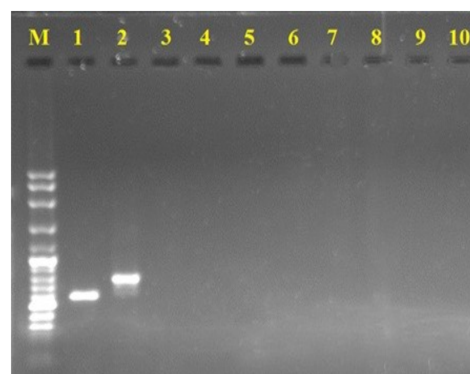

Figure 2. PCR product employing primer pairs *Stx1* and *Stx2*. M: 100 bp DNA marker (Vivantis), lane 1 and 2: *E. coli* O157 ATCC 43894 as positive control (lane 1: *Stx1* gene, 614bp, lane 2: *Stx2* gene, 779bp), lane 3 and 4: negative control (*E. sakazakii*), lane 5 and 6: sample 1, lane 7 and 8: sample 2, lane 9 and 10: sample 3.

4. Discussion

A 30 raw milk samples taken from Sleman district, Yogyakarta, found out 11.428 colonies that were negative sorbitol. Members of the *En-*

terobacteriaceae that lacking ß-glucoronidase but not ß-galaktosidase are like *Escherichia coli* O157, *Enterobacter*, *Citrobacter* and *Klebsiella* appear as salmon to red colonies on CCA.

They were screened on the selective medium Chromagar O157 to confirmed negative sorbitol specially for *E. coli* O157. Confirmation test of 3 Isolates negative sorbitol by serological test shows no agglutination. This result shown that they didn't have somatic cell O157, so they are not *E. coli* O157 strain.

Detection of toxin genes *Stx1* and *Stx2* was done by using single primer pair found there is no Stx1 and Stx2 detected from 3 suspected isolates.

PCR is considered to be the most sensitive means of determining whether a fecal specimen or a food sample contains STEC. Although direct extracts of feces or foods could be used as templates for PCR, the best results are usually obtained by testing extracts of primary broth cultures. Broth enrichment serves two purposes: inhibitors in the sample are diluted, and bacterial growth increases the number of copies of the target sequence [7,12,9].

STEC related to severe disease such as haemorrhagic colitis, haemolytic uraemic syndrome, thrombotic thrombocytopenic purpura, and acute kidney failure [19,6,21]. Thus, any food that human consumed must be free from STEC *E. coli* O157 and other O antigen [12]. To checked the presence of *Stx* genes, mPCR could be used by combining different primer pairs for *Stx1* and *Stx2* or use the single pair (*Stx1* or *Stx2*) [12,9]. It is reported that PCR assay was able to identify virulence-specific genes such as *Stx1*, *Stx2*, *eaeA*, and entero-hemorrhagic *E. coli* Hemolysin (EHEC hlyA) [2,9].

From all the finding that we had, it's not in agreement with previous results that found the presence of *E. coli* O157 in Yogyakarta, Bogor, and Bali [18,16,1,17].

5. Conclusions

This research proof that raw cow's milk in the sample which collected from Sleman are free from *E. coli* O157 and its genes-encoding toxin, *Stx1* and *Stx2*.

References

[1]Agus, Made Hendrayana, Komang Januartha Putra Pinatih, Amy Yelly. Deteksi bakteri *Escherichia coli* Serotipe O157 pada daging babi di Kota Denpasar. Jurnal Ilmiah Kedokteran Medicina 2012; 43: 3-8.

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

[3]Budiarso TY. Teknologi Deteksi *Verocytotoxigenic Escherichia coli* (VTEC). "Prospek bisnis E-Medicine dan Corporate Medicine dan hubungannya dengan teknologi pendidikan kedokteran" Proseeding Seminar Prospek Bisnis E-MedicineISBN 979.8139.79.8 2005:40-51

[4]Centers for Disease Control and Prevention. Investigation Announcement: Multistate Outbreak of *E. coli* O157:H7 Infections Linked to Romaine Lettuce. USA 2011.

[5]Centers for Disease Control and Prevention. 2012. Multistate Outbreak of Shiga Toxin-producing *Escherichia coli* O157:H7 Infections Linked to Organic Spinach and Spring Mix Blend. USA

[6]Fox, Alvin. Enterobacteriaceae, Vibrio, Campylobacter and Helicobacter, Bacteriology-Chapter 11. Microbiology and Immunology Online. University of South Carolina School of Medicine 2011.

[7]Gannon VP, King RK, Kim JY, Thomas EJ. Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Appl. Environ Microbiol 1992; 58: 3809–3815.

[8]Hall, William F. An assessment of available information on raw milk cheeses and human disease 2000–2010. MAF Technical Paper. Wellington 2011.

[9]Jalil, K, Vadood R, Abolfazi B. Direct detection of *Escherichia coli* O157 and its major virulence factor genes in animal faeces at slaughter using multiplex polymerase chain reaction (PCR). Afr J Microbiol Res 2011; 5(14): 1763-1767.

[10]Jamshidi, Bassami MR, Rasooli M. Isolation of *Escherichia coli* O157:H7 from ground beef samples collected from beef markets, using conventional culture and polymerase chain reaction in Mashhad, northeastern Iran. Iranian Journal of Veterinary Research, Shiraz University, 2008;9 (1): 72-76.

[11]Law, Derek. The history and evolution of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. World Journal of Microbiology & Biotechnology 2000; 16:701-709

[12]Paton, Adrienne W, James C, Paton. Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR Assays for *Stx1*, Stx2, eaeA, enterohemorrhagic *E. coli*

hlyA, rfbO111, and rfbO157. J. Clin Microbiol 1997;36:598–602.
[13]Petridis H. *E. coli* O157:H7 A Potential Health Concern. Institute of Food and Agricultural Sciences, University of Florida, USA 2002.
[14]Robert L, Buchanan, Doyle MP. Foodborne Disease Significance of *Escherichia coli* O157:H7 and Other Enterohemorrhagic *E. coli*. Institute of Food Technologists, USA. 1997.
[15]Sambrook J, Fritsch EF, Maniatis T. Molecular cloning : a laboratory manual. 2nd Edition. Cold Spring Harbor Laboratory Press. New
[16]Suardana ,Wayan I. Analisis Keanekaragaman Genetik dan Struktur Protein Shiga Like Toxin (Stx) *Escherichia coli* O157:H7 Isolat Lokal Sebagai Dasar Pengembangan Kloning Gen untuk Pembuatan Antibodi Monoklonal. UGM, Yogyakarta 2009.
[17]Suardana ,Wayan I, Sujaya IN, Artama WT. Aplikasi Kandidat Pemindai untuk Diagnosis Gen Shiga like toxin-2 dari *Escherichia coli* O157:H7. Jurnal Veteriner 2012;13(4): 434-439.
[18]Sudrajat, Dadang, Maria LR, Suhadi F. Deteksi cepat bakteri *Escherichia coli* Enterohemoragik (EHEK) dengan metode PCR (Polymerase Chain Reaction). Risalah Pertemuan Ilmiah Penelitian dan Pengembangan Teknologi Isotop dan Radiasi. Puslitbang Teknologi Isotop dan Radiasi, Batan, Jakarta 2000.
[19]Tahamtan YE,, Pourbakhs SA, Shekarforoush SS, PCR detection of *Escherichia coli* O157:H7 directed from slaughtered cattle in Shiraz, Iran. Razi vaccine and serum research institute. Iran 2006.
[20]Takkinen J. Shiga toxin/verotoxin-producing *Escherichia coli* in humans, food and animals in the EU/EEA, with special reference to the German outbreak strain STEC O104. Stockholm: ECDC. 2011
[21]WHO (World Health Organization). Enterohaemorrhagic *Escherichia coli* (EHEC). http://www.who.int/mediacentre/factsheets/fs125/en/ 2011