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Monitoring of Pollution of *Salmonella* sp. in Raw Milk Using Virulence Gen Marker

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Abstract

Salmonella sp. is an enteropathogenic organism and it can spread through contaminated food which is rich in nutrition such as raw milk. The goal from this research is to find out the suitable pasteurization and time effect on the growth isolates *Salmonella* sp. that its *inv*A presence is detected. Seventy-five (75) samples were taken in three locations of milk collection from groups of breeders. 10 ml of milk samples were grown in enrichment culture of modified Tryptic Soy Broth (mTSB) at 37^{o}C for 12 hours. Cellular culture was then grown in differential selective medium of Salmonella Shigella Agar (SSA) and Chromocult Coliform Agar (CCA). From 75 milk samples produced 1392 colonies of typical *Salmonella* sp. pathogens. Then, isolates were selected using Triple Sugar Iron Agar (TSIA) and Urea Broth media, producing 3 suspected isolates of *Salmonella* sp which was known from colonies color. Results of confirmation using API 20E and 50 CHE produced two isolates of *Salmonella* spp. with %ID = 99.6% and one isolate of *Salmonella typhi* with %ID = 72.1%. All of the isolates were then detected for virulence factor using invA marker and all positive isolates have virulence factor. It indicates that raw milk contains polluted pathogenic bacteria.

Keywords: raw milk, enteric pathogenic, virulence gen

Introduction

Outbreak due to consuming milk and its products in the period 1998-2010 as reported by the Centers for Disease Control and Prevention's (CDC) have been caused 2,824 people sick and 8 died. This extraordinary event was caused by contaminated milk in most groups of pathogenic enteric bacteria such as Salmonella, Escherichia coli, Yersinia enterolitica, Campylobacter spp., Staphylococcus aureus, Clostridium perfringens, Bacillus cereus, Listeria spp. and Shigella spp. (CDC 2012, CDC NORS 2012, Langer et al. 2012). In the period 2010-2012 this outbreak experienced an increase caused by E. coli contamination producing shiga toxin and Salmonella enterica serotype Typhimurium (Mungai, et. al., 2015).

Yogyakarta has dairy centers producing uncooked milk distributed to milk processing industries and some are sold as fresh milk products by street sellers and cafes existing in Special District of Yogyakarta. The previous studies had found contaminations of Salmonella spp. and Salmonella typhi in fresh milk products (Budiarso & Prihatmo, 2015). The contamination of pathogenic enteric bacteria in milk must be controlled considering

that these bacteria can danger human's health. These family of enteric bacteria stood on the highest reputation as disease-causing bacteria which are generally associated with the intestine pathway. Various cases of pathogenic enteric bacteria contamination in food and beverages have caused serious health problems in the world. Although some genera include normal flora in the human digestive tract such as Escherichia, Enterobacter and Klebsiella, some groups of the enteric bacteria have some pathogenic grooves in human such as Salmonella, Shigella, Yersinia, Enterobacter sakazakii and E.coli O157:H7 (Doyle and Schoeni 1987, Ray and Bhunia 2011). The Salmonella genus is reported to cause an average of 500 cases of salmonellosis. One of the causative agent is Salmonella typhi which was found pathogenic and it used human as its specific host. This infection of bacterium occurs in fecal-oral through food and beverages contaminated by Salmonella typhi (Ray and Bhunia, 2011).

Contamination of enteric pathogenic bacteria in raw milk is difficult to avoid. It can be happened because enteric bacteria living in digestive tract in both animal and human causes contamination when going out with feces to environment. In dairy cow, feces containing this bacteria pollution can bind to the cow body, especially in areas difficult to wash, such as, from fold area of thigh to body back, tail, udder, and nipple. This condition becomes main source of contamination because the bacteria can enter into milk during dairy process. Contamination may also occur due to poor pen sanitation, workers' cleanliness, milk container/ collector, storage media and transportation instruments (Forsthy and Hayes, 1998; Ray and Bhunia, 2011). Results of field survey indicate that the contamination factors are frequently found in breeding zones available to areas of Special District of Yogyakarta. This study objective is to explore enteric pathogenic bacterium in raw milk deriving from groups of breeders in Special District of Yogyakarta.

Materials and Methods

The materials used in this study were samples of raw cow's milk from a group of farmers in three different milk storage locations in the Special Region of Yogyakarta. Sampling was done during daytime of milking, which one sample in each location was taken every day for 25 days so that the total number of samples is 75. Then, the samples were entered into sterile bottles, and stored in ice box and analyzed in laboratory in less than two hours. The methods for obtaining pathogenic enteric bacterial isolates from the sample were as follows: (1) Enrichment culture, which was carried out in accordance with FDA-BAM procedure (2011) using Novobiocin Tripticase Soy Broth (mTSB) medium containing 20 mg/Ll Novobiocin (Doyle and Schoeni 1987, AOAC 1995). (2) Selection of pathogenic enteric bacteria using differential selective medium Chromogenic Coliform Agar (CCA) for growing pathogenic enteric bacteria colonies. Pathogenic colonies give a clear and bright blue appearance, and subsequently selected using Salmonella Shigella Agar (SSA) medium, Triple Sugar Iron Agar (TSIA) and Urea Broth to ensure suspected pathogens such as Salmonella, Shigella and Yersinia (Turner, et al., 2000; Budiarso & Prihatmo, 2015). (3) Suspected colonies enteric pathogen were tested with Biochemical characterization using API 20E kits, and API 50CHE Biomerieux (Taskilla et al., 2012; Chajecka et al., 2003; WHO 2003). and (4) Salmonella sp. identified isolates then continued molecular characterization using PCR with invA virulence gen marker. Identified isolates of Salmonella sp. were then characterized molecularly using PCR. In the first stage DNA isolation was carried out using PureLink® Genomic DNA Kits. Isolates

were grown in 3 ml of BHIB (Brain Heart Infusion Broth) medium at 37°C for 18-24 hours. After that, the isolate was poured in a 1.5 ml tube and centrifuge with a speed of 10,000 rpm for 3 minutes. The supernatant was removed, then the pellet was washed with 180µL PureLink® Genomic Digestion Buffer and added 20 µL of proteinase K then homogenized using vortex. Furthermore, it was incubated in a 55°C bath for 30 minutes to 4 hours and then added 20 mL of RNase A were mixed quickly then incubated at room temperature for 2 minutes. The sample was added 200µL of PureLink® Genomic Lysis / Binding Buffer and mixed until homogeneous, added with 200µL 96-100% ethanol and mixed again until homogeneous and then poured into PureLink® Spin Column then centrifuged at 10,000 rpm for 1 minute. The collection tube on the bottom is then discarded and replaced with a new one, then 500 ml of Wash Buffer 1 is added and centrifuged again at 10,000 rpm for 1 minute. The lower collection tube replaces it with a new one, after that is added 500µL Wash Buffer 2 and then centrifuged again at a speed of 13,000 rpm for 3 minutes then the lower collection tube is removed again. Colomn spin was placed into 1.5 mL sterile tube, then added (25-200) µL ddH2O and incubated at room temperature for 1 minute then centrifuged at 13,000 rpm for 10 minutes. After that, pure DNA is stored in the refrigerator at -20°C. The product was electrophoresis using 0.8% agarose gel and SYBR Safe solution with 0.1% concentration and then visualized with UV lighting. Molecular testing was carried out by detecting the presence of virulence genes using S139-S141 specific primers with nucleotide sequences based on inva gene markers 5'GTG AAA TTA TCG CCA CGT TCG GGC AA-3 'and 5' TCA TCG CAC CGT CAA AGG AAC C-3 '. The condition of PCR uses 35 cycles of amplification with the pre-denaturation stage using a temperature of 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes (De clercq, 2007; Rahn et al., 1992; Gunaydin, 2007; Malomy, et al., 2003).

Results and Discussion

Enteropathogenic bacteria were obtained by growing 10-ml milk samples into enrichment culture modified Tryptic Soy Broth (mTSB) medium containing 20-mg/L novobiocin and bile salts playing role in inhibiting positive gram bacterial group and non-intestine bacteria. Bacteria belonging to enteric pathogenic are Salmonella, Shigella, Yersinia and Escherichia coli 0157:H7. Using this medium, all enteric pathogenic groups could grow well (Kanki et.al., 2011). Bacterial culture from enrichment stage was enumerated in Chromocult Coliform Agar (CCA) medium. Chromocult Coliform Agar (CCA) medium is differential selective medium containing chromogenic substrates giving different colony appearances to each member of family Enterobacteriaceae. Bacterial group of genus Citrobacter, Enterobacter, and Klebsiella would give red colony appearance because this bacteria are able to use β-galactosidase enzyme substrate but these cannot use β-glucuronidase enzyme substrates. Bacterium of Escherichia coli able to use β-galactosidase and β-glucuronidase enzyme substrates would be dark blue or violet. Meanwhile, enteric pathogenic groups of Salmonella, Shigella, Yersinia and Proteus unable to use β-galactosidase enzyme substrate but able to use β-glucuronidase enzyme substrate would be light blue or white (Turner et al., 2000). The results milk sample enumeration are shown in Table 1.

Table 1. Results of colony enumeration suspected as enteric pathogen of *Salmonella* streak plate in raw milk samples

Location of Collection	Enumeration of enteric pathogen in CCA medium (light blue /white) [in colonies]	Selection of *Salmonella* sp (*black center*) in SSA medium [in colonies]	TSI Test (+) [in colonies]	Urease test (-) [in colonies]	∑ Positive suspected as *Salmonella* sp [in colonies]
25 sample Group-1	447	17	1	1	1
25 sample Group-2	731	3	1	1	1
25 samples Group-3	514	19	1	1	1
Total	1692	39	3	3	3

Colony suspected as suspected Salmonella giving clear white and light blue color appearances was then selected by streak plate 6 radian in SSA medium. Typical colony Salmonella streak plate in SSA medium has typical colorless black center because its ability to produce H2S (Takskilla et al., 2012). Isolates suspected as Salmonella streak plate were then selected furthermore through biochemical test using TSIA. TSIA medium functions as sulfur source to see production of H2S gas because its existence with reaction between ferric ammonium citrate and Fe and sulfide forms black color of ferro sulfite compound and use of glucose for producing gas. Several parameters examined were based on the Indonesian Nasional Standard (Standar Nasional Indonesia/SNI) No. 01-3141-1998 for Fresh Dairy Milk and SNI No. 7833-2009, such as total bacteria and coliform.

Positive isolates were then selected again into urea broth medium to detect the ability for degrading urea. Salmonella sp. was unable to degrade urea or carbon and nitrogen bond in amide bond forming ammonium alkaline in end product because it did not have urease enzyme.

Based on colony growth, colony was suspected as Salmonella sp. of 75 milk samples producing 192 colonies and then selected in SSA medium generating 39 isolates which were then selected furthermore in H2S and urease test only generating 3 isolates. Three isolates were suspected as Salmonella and then confirmed biochemically using API test. Test used API system being a test based on changes in media already inoculated by bacterial test where the results were inputted into API Web Biomeriux. Characteristics of inputted test bacteria were processed by system to analyze complying with database of Biomeriux system so that results of identification were obtained in %ID (Index Determination). API 20E system is a set of microbe stripes containing carbon sources such as ONPG, ADH, LDC, ODC, CIT, H2S, URE, TDA, IND, VP, GEL, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, and ARA. This system is used to identify characters of each bacterial group in a family of Enterobacteriaceae. The results of specificity and sensitivity test of API 20E test in isolate 123, Salmonella enterica isolate, and member of Enterobacteriaceae family were 99%. The results of suspected Salmonella sp. test using API 20E and API 50 CHE of three isolates, two of them were identified as Salmonella spp. with %ID = 99.6% and one isolate was identified as Salmonella typhi with %ID = 72.1% as shown in the Figure 1 below (Holmes et al., 1978; Nucera et al., 2006).

Very Good Identification				
Strip	API 50 CHE V3.1			
Profile	-+--+++---++++-++-++--+--+--+-+-+----------+--+-+-+++++-----			
Note	Confirm by serological tests			
Significant taxa	**%ID**	**T**	**Test against**	
Salmonella spp	99.6	0.88	ESC 3%	
Next taxon	**%ID**	**T**	**Test against**	
Salmonella typhimurium	0.3	0.58	ESC 0%	TAG 100%

Figure 1. Results of suspected *Salmonella* sp. isolate confirmation using API 20E and 50 CHE

Detection of virulence genes used invA marker in isolate identified as Salmonella sp. using specific primer method of S139-S141 with nucleotide sequence based on invA gen markers of 5'GTG AAA TTA TCG CCA CGT TCG GGC AA-3' and 5' TCA TCG CAC CGT CAA AGG AAC C-3' (Rahn, et al., 1992). The results of virulence gene amplification used as invA gen marker are shown in Figure 2. DNA amplification using invA has been recognized as international standard to detect Salmonella sp. because amplification of 99.4% is accurate and specific, able to identify strain of Salmonella of invA gen, this gen codifies protein in Salmonella membrane responsible for invasion in epithelial cells (Karmi, 2013). Positive control used was Salmonella typhi NCTC 786 and negative control used was Escherichia coli. The results of study indicated that isolates of Salmonella Kp 1, Kp 2 and Kp 3 had results similar to control of Salmonella typhi NCTC T86 in 284 bp, Escherichia coli was not detected because invA is specific primer for Salmonella.

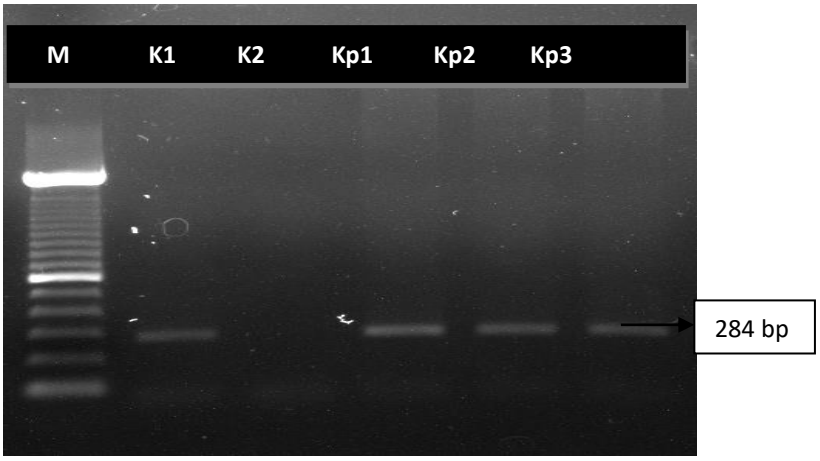

Figure 4. Results of *inv*A primer DNA amplification of isolates of Kp 1, Kp 2, and Kp 3
Where: M = marker, K1 = *Salmonella typhi* NCTC 786, K2 = *Escherichia coli*, Kp 1 = Breeder Group 1, Kp 2 = Breeder Group 2, Kp 3 = Breeder Group 3.

These results can be found furthermore using sequencing or comparing to Salmonella nucleotide database. Salmonella having invA gen because it is able to invade small intestine mucosa, developing in epithelial cells and producing toxin to cause inflammatory reaction and liquid accumulation in intestine. Salmonella exists in epithelial cells, self-developing, and self-producing thermolabile enterotoxin, directly affecting water and electrolyte secretion so that it is potential to cause pain (Ray and Bhunia, 2011).

Conclusion

The results of monitoring the pathogenic enteric bacteria contamination in raw milk in a group of farmers in Yogyakarta Special Region found three isolates of pathogenic batteries. These isolates were identified as *Salmonella* spp with% ID (96.6%) as much as two isolates and one isolate as *Salmonella typhi* with% ID (72.1%) based on the results of confirmation using API 20E and API 50 CHE. The three isolates had virulence factors based on *inv*A gene detection which showed the three isolates were pathogenic.

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