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Risk of *Escherichia coli* O157:H7 transmission linked to the consumption of raw milk

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Abstract

E. coli O157:H7 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 investigate the prevalence of *E. coli* O157:H7 in raw cow, goat and buffalo milk samples. MPN-PCR method targeting the major virulence *rfbE* gene and *fliCH*$_7$ gene of *E. coli* O157:H7 was used. Total of 177 raw milk samples were collected from local dairy farms in the state of Selangor, Malaysia. The highest prevalence of *E. coli* O157:H7 was found in raw cow milk (8.75%) followed by raw goat milk (7.32%) and raw buffalo milk (1.79%). The estimated quantity of *E. coli* O157:H7 in raw cow, goat and buffalo milk ranged from <30 MPN/g to 120 MPN/g. In raw cow and goat milk samples examined contain *E. coli* O157:H7 microbial load ranged from 30 to 120 MPN/g and 30 to 36 MPN/g, respectively. *E. coli* O157:H7 microbial load in buffalo milk samples was found to be the lowest, only 30 MPN/g. Results of this research provide useful information on biosafety of *E. coli* O157:H7 in raw milk marketed in Malaysia.

Introduction

Food-borne diseases are important public health and economic burden (Sockett, 1993; Djuretic *et al*., 1996; Schlundt *et al*., 2004) and result in considerable morbidity and mortality rate (Morse *et al*., 1994; WHO, 1998). Exposure of processed food to contaminated raw material increases the chance of cross-contamination. Other factors associated with cross-contamination and food-borne illnesses are improper food processing, cooking, storage and handling (Panisello *et al*., 2000). According to WHO (2001), 2.1 million people (mostly children) die in less develop countries yearly due to food-borne and water-borne diarrheal diseases. Only food-borne diarrhea is responsible for 1.8 million deaths among children every year (WHO, 2007c). Problems associated with food-borne disease are vomiting, diarrhea, liver and kidney failure, neural and brain disorders and in the most severe cases, death. Long term problems related to food-borne disease are paralysis and reactive arthritis (Schlundt, 2002).

It has been reported that 76 million cases of food-borne diseases occur annually in US, resulting in 325,000 hospitalizations and 5000 deaths (Mead *et al*., 1999). Extrapolating the US data on food-borne diseases to the rest of the world, Ka¨ferstein and Abdussalam (1999) reported that almost one third of the population in developed countries is affected with food-borne illness annually. The incidence of food-borne deseases is more likely to be higher in developing countries. In 2009, water-borne and food-borne diseases were reported in Malaysia (ranging 0.14 - 1.07 cases per 100,000 populations). Food poisoning cases had the highest incidence rate of 62.47 cases per 100,000 populations in 2008 and 36.17 cases in 2009 (MOH, 2009, 2010a). As there is little effort to reveal the impact and exact significance

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of food-borne disease, the true incidence of food-borne outbreaks in Malaysia is still unknown (Lim, 2002). Moreover, insufficient investigation on food-borne disease in most of the developing countries is the main reason for undetected cases in food-borne outbreaks (Beuchat, 1998).

During an outbreak investigation of hemorrhagic colitis in 1982, *Escherichia coli* O157:H7 was first considered as a pathogen. In 1993 large multistate outbreaks in US brought *E. coli* to the forefront (Rangel *et a*l., 2005) and it was recognized as one of the most important and virulent food-borne pathogens in 1996 (Li *et al*., 2011). *E. coli* O157 infection results in diarrhea, thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (Wells *et al*., 1991; Bleem, 1994; Coia *et al*., 2001). HUS is characterized by renal injury, thrombocytopenia and hemolytic anemia (Rangel *et al*., 2005).

Due to the low infective dose, severity of the disease symptoms and the case fatality rate, *E. coli* O157:H7 is considered a harmful threat in food safety (Kaper *et al*., 2004; Meng *et al*., 2007). *E. coli* O157 contaminates water and food products. Milk is considered a high risk food as it is highly nutritious and serves as an ideal medium for bacterial growth (Chye *et al*., 2004). Dairy farms act as reservoirs for several food-borne pathogens such as Shiga-Toxin producing *Escherichia coli* (STEC), *Listeria*, *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter* and *Salmonella* (Oliver *et al*., 2005; Aidar-Ugrinovich *et al*., 2007; Vimont *et al*., 2007). Dairy cattle are the major source of STEC strains in milk that contaminate milk and meat through direct contact with the cattle and the dairy farm environments (Nataro and Kaper, 1998; Hussein and Sakuma, 2005). STEC strains are highly pathogenic to human with low infectious dose, causing food-borne disease through consumption of contaminated water or food.

Raw milk is known as the main transmission pathway for pathogens resulting in food-borne outbreaks every year (Potter *et al*., 1984; CDC, 1999; Gillespie *et al*., 2003; Rey *et al*., 2003; Chye *et al*., 2004). Raw milk exposed to untreated and contaminated water, cattle or human faeces can easily be contaminated with *E. coli*. Unpasteurized milk and dairy products made from raw milk (such as soft cheese) act as vehicles for transition of *E. coli* to human (Dweik *et al*., 2012). Consumption of raw milk is a high risk behavior leading to morbidity and mortality (Keene, 1999). Generally, raw milk consumption is a traditional practice among farm families (Jayarao *et al*., 2006). Raw milk consumers claim that raw milk is healthier, although equal nutritional value of raw and pasteurized milk has been proved (Potter *et al*., 1984; Centers for Disease Control and Prevention, 1999; Bren, 2004).

Milk is an important component in infants and elderly daily diet. As the immune system of high risk groups (children, elderly and pregnant women) is extremely susceptible to pathogens (Ryser, 1998), special attention must be paid to the microbiological safety of milk. The aim of this study was to investigate prevalence of *E. coli* O157:H7 in raw milk samples.

Materials and Methods

Sample collection

A total of 177 raw milk samples including raw cow milk (80 samples), raw goat milk (41 samples) and raw buffalo milk (56 samples) were collected from 3 local dairy farms in the state of Selangor, Malaysia. Samples were kept on ice (during collection and transportation) and analyzed immediately.

Most Probable Number (MPN)

Ten ml of raw milk sample was added with 90 ml of Trypticase Soy Broth (TSB) in a sterile stomacher bag and homogenized for 30 sec. The homogenized sample was incubated at 37°C for 24 h. Three tubes MPN analysis performed using serial dilution from TSB enriched samples. One ml of each dilution was transferred into three sterilized MPN tubes and incubated at 37°C for 24 h.

DNA extraction

Boil cell method was used for DNA extraction. Each MPN tube was centrifuged for 2 min at 12,000 rpm and the supernatant was discarded. TE buffer (500 µl) was added to each tube and boiled for 10 min at 100°C. The boiled cell lysate was immediately cooled at -20°C for 10 min. The mixture was centrifuged at 12,000 rpm for 3 min and supernatant was used for PCR amplification.

Multiplex polymerase chain reaction (mPCR)

PCR amplification for detection of *E. coli* O157:H7 was performed in 25 µl reaction mixture containing 5 µl of 5X PCR buffer, 0.5 µl of 10 mM deoxynucleoside triphosphate mix, 2 µl of 25 mM $MgCl_2$, 1.25 µl of 0.5 µM of *rfbO157* primer, 2.5 µl of 1 µM *fliCH*$_7$ primer, 0.2 µl of 5U *Taq* polymerase, 7.8 µl of distilled water and 2 µl of DNA extract. Two specific primer pairs, the *rfbO157* primer with forward sequence 5'- CGG ACA TCC ATG TGA TAT GG -3' and reverse sequence 5'- TTG CCT ATG TAC AGC TAA TCC -3' and the *fliCH*$_7$ primer with forward sequence 5'- GCG CTG TCG AGT TCT

ATC GAG-3' and reverse sequence 5'- CAA CGG TGA CTT TAT CGC CAT TCC -3' were used to produce amplicons of 259 bp and 625 bp targeting the major virulence *rfbE* gene and *fliCH*$_7$ gene of *E. coli* O157:H7, respectively. The following thermal cycling conditions was used: pre-denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 2 sec, annealing at 55°C for 1 min, extension 72°C for 1 min and final extension of 72°C for 10 min. The PCR products were subjected to gel electrophoresis using 1.5% agarose gel with 0.5X Tris-borate-EDTA (TBE) buffer at 100V for 32 min. A 100 bp ladder was used as the DNA size marker and the gel was visualized under UV light using the Gel Documentation System (SynGene, USA).

Results and Discussion

Prevalence of *E. coli* O157:H7 in 177 raw milk samples was investigated using MPN-PCR method. Results of gel electrophoresis for targeted *rfbE* and *fliCH*$_7$ genes at 259 bp and 625 bp, is showed in Figure 1. The highest prevalence of *E. coli* O157:H7 was found in raw cow milk (8.75%) followed by raw goat milk (7.32%) and raw buffalo milk (1.79%) (Table1). The estimated quantity of *E. coli* O157:H7 in raw cow, goat and buffalo milk ranged from <30 MPN/g to 120 MPN/g. The highest quantity of *E. coli* O157:H7 detected in raw cow, goat and buffalo milk was 120 MPN/g, 36 MPN/g and 30 MPN/g, respectively. Other researchers have reported *E. coli* in raw milk, commercial ice cream bars, cheese curds and butter made from raw milk (Rangel *et al*., 2005) and pasteurized milk (Li *et al*., 2011). Rey *et al*., (2006) reported that 0.3% of caprine and ovine milk samples collected from the bulk tank were contaminated with *E. coli* O157:H7. They detected STEC strains in 10.8% of caprine and ovine milk samples from the bulk tank, 5% of cheese and 3.9% of fresh cheese curds produced in Extremadura (Western Spain). No Verotoxin-producing *Escherichia coli* (VTEC) was found in raw goat milk samples collected from bulk tank in Murcia region (Southeastern Spain) (Corte´s *et al*., 2005). The result variation in microbiological quality of raw milk in different studies may be due to disparate sample size, isolation, and identification method, farm size, geographic area, number of animals on the farm and farm management practices (Jayarao *et al*., 2006).

According to Greig (2010), even low dose of *E. coli* O157:H7 (10 to 100 CFU) is sufficient to cause infection and the infectious dosage for children is only 1 to 4 CFU (Duncan and Hackney, 1994). Based on the risk assessment study using Risk Ranger, the estimated risk ranking for *E. coli* O157:H7 infection related to consumption of raw milk is 73, indicating that there is risk of *E. coli* O157:H7 infection even though the prevalence detected is relatively low.

Table 1. Prevalence of *Escherichia coli* O157:H7 and its microbial load (MPN/g) in raw milk samples detected and quantified by combined MPN-PCR method

Type of milk	Total samples (n)	Number of positive/ Prevalence (%)	Microbial loads (MPN/g)		
			Min[a]	Med[b]	Max[c]
Cow	80	7 / 8.75	<30	61	120
Goat	41	3 / 7.32	<30	36	36
Buffalo	56	1 / 1.79	<30	<30	30

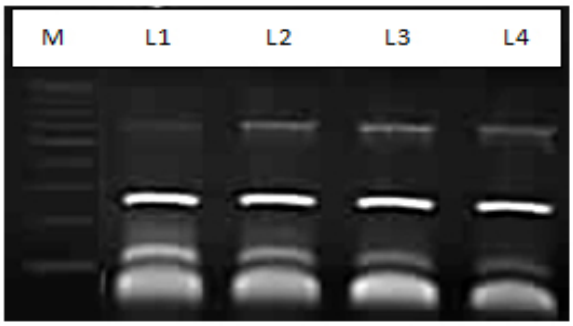

Figure 1. PCR amplification of *rfbO157* and *fliCH*$_7$ genes producing amplicon at 259 bp and 625 bp, respectively. M: Molecular marker, ladder 100 bp; L1: Positive control; L2-L5: Positive samples

The results showed that all three types of raw milk samples were contaminated with *E. coli* O157:H7. Several factors contribute to milk contamination such as poor hygienic milking conditions, contaminated equipments, milking utensils and milk handlers' poor personal hygiene (Olson and Mocquot, 1980; Cousin, 1982). Ayres *et al*. (1980) suggested that bacteria may enter into raw milk from the external surface of udder. Chye *et al*. (1994) concluded that unhygienic milking practices and milk storage at elevated temperature may increase number of microorganism in milk. According to O' Connor (1994), safe milk is produced from healthy cows, where milking process is performed under hygienic conditions and milk contains less than 5.0 x 10^4 bacteria per ml.

In observation of small farms in Peninsular Malaysia, unsatisfied level of hygiene was found. Cattle feces were observed around the farm floor and attached to the milking and feeding equipments. Poor hygiene of feeding equipments and contaminated feed are playing an important role in milk contamination. According to Oksuz *et al*. (2004) most of the *E. coli* O157:H7 outbreaks were associated with cattle feed origin especially those contaminated with cattle feces. Mead and Griffin (1998), found *E. coli* O157 in healthy cattle feces. To avoid raw milk contamination,

hygiene conditions of farm must be improved.

Conclusion

Detection of *E. coli* O157:H7 in raw milk samples revealed that raw milk is a serious public health concern since less educated people and farm families still consume milk without further heat processing. Effective and continuous training accompanied with emphasize on the safety and health issues related to raw milk hazards is really needed. To ensure the quality of raw milk, everyone engaged in milk and dairy production line (from dairy farms to the dairy food processing units) should be trained for hygienic practices. Newly emerging food-borne pathogens, new characteristics among existing pathogens and unpredicted new food vehicles are questions and problems for food industries and public health agencies. To protect public health, more stringent regulations and strategies are in demand.

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