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BOX-PCR and ERIC-PCR evaluation for genotyping Shiga toxin-producing *Escherichia coli* and *Salmonella enterica* serovar Typhimurium in raw milk

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Abstract:
Over the past decade, the occurrence of milk-borne infections caused by Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) has adversely affected consumer health and the milk industry.
We aimed to detect and genotype the strains of *E. coli* and *S.* Typhimurium isolated from cow and goat milks using two genotyping tools, BOX-PCR and ERIC-PCR. A total of 200 cow and goat milk samples were collected from the dairy farms in Southern Sarawak, Malaysia.
First, *E. coli* and *Salmonella* spp. detected in the samples were characterized using PCRs to identify pathogenic strains, STEC and *S.* Typhimurium. Next, the bacterial strains were genotyped using ERIC-PCR and BOX-PCR to determine their genetic relatedness. Out of 200 raw milk samples, 46.5% tested positive for non-STEC, 39.5% showed the presence of *S.* Typhimurium, and 11% were positive for STEC. The two genotyping tools showed different discrimination indexes, with BOX-PCR exhibiting a higher index mean (0.991) compared to ERIC-PCR (0.937). This suggested that BOX-PCR had better discriminatory power for genotyping the bacteria.
Our study provides information on the safety of milk sourced from dairy farms, underscoring the importance of regular inspections and surveillance at the farm level to minimize the risk of *E. coli* and *Salmonella* outbreaks from milk consumption.

Keywords: Food safety, epidemiology, public health, *Escherichia coli*, *Salmonella* spp., milk-born infections, genotyping

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INTRODUCTION

Escherichia coli is generally known as normal microflora in the intestines of birds and mammals. However, not all of its strains are commensal to humans. Enteric *E. coli* is clustered into six pathotypes based on its pathogenicity profiles (virulence factors, clinical manifestation, and phylogenetic profile). They are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) [1]. Among many *E. coli* pathogenic strains, *E. coli* O157:H7 is the most notable serotype associated with food poisoning [2]. *E. coli* O157:H7 is one of EHECs that harbors and expresses the genes for Shiga toxins type 1 (*Stx1*) and 2 (*Stx2*) that result in hemorrhagic colitis (HC) in humans. A life-threatening sequel of hemorrhagic colitis is hemolytic uremic syndrome (HUS). *Salmonella* is a causative agent of severe foodborne disease worldwide, with most of the infections caused by *Salmonella enterica* [3, 4]. The symptoms of salmonellosis are headache, fever, diarrhea, nausea, vomiting, and abdominal cramp. These symptoms usually start 12 to 72 h after the ingestion and can last up to four to seven days, depending on the

severity of the infection. Infants, the elderly, and immune-compromised groups are generally more susceptible to salmonellosis [5]. In Malaysia, it is difficult to evaluate the status of salmonellosis due to the lack of detailed epidemiological studies by the public health and veterinary sector.

Molecular typing is crucial in studying outbreaks, identifying transmission routes, detecting pathogen cross-transmission, and determining sources of infection [6]. The enterobacterial repetitive intergenic consensus (ERIC)- and the BOX repetitive sequence (BOX)-polymerase chain reaction (PCR) are examples of genotyping tools. We used these tools to discriminate the strains of *E. coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), as well as investigate their power in clustering according to the origin of bacterial isolates.

The BOX-PCR was first used for the genetic characterization of *Streptococcus pneumoniae*. It produced amplicons based on repetitive sequences in the bacterial genome and was later used to discriminate many bacterial species [7]. This tool employs the BOX A1R primer for the repetitive element sequence-based PCR to amplify the repetitive regions of the bacterial genome. This primer has been found in many microbial genomes in previous studies. The band profiles of the amplified repetitive regions are unique among the species or even between the species. Thus, different species can be identified through their band patterns [8].

The ERIC-PCR is commonly used for the genetic characterization of *E. coli.* It is more powerful than other molecular fingerprinting tools such as PCR ribotyping, RAPD-PCR, or PFGE [9, 10]. Its other advantages include fast speed, sensitivity, and reliability [11]. BOX sequences are highly conserved, but their chromosomal locations differ between the species [7]. Thus, ERIC sequences are used in the PCR as practical primer binding sites to produce fingerprints of different bacterial genomes. They differ from the sequences from other bacterial repeats assays (e.g. BOX-PCR) due to their more comprehensive species distribution range [12].

Raw milk is defined as milk that has not been processed, e.g., via pasteurization and homogenization. Unprocessed milk is perceived to have more nutritional benefits than processed milk. Consumers believe that foods in their natural and unprocessed form are safer and healthier, although such beliefs have not been proven and remain the subject of ongoing debate [13]. The practice of raw milk consumption is prevalent and linked to consumers' educational level, socioeconomic factors, and living on dairy farms. This is of public health concern since there is an 850 times higher risk of acquiring infections from consuming raw dairy products compared to pasteurized milk [14]. Thus, the risk of infection by pathogenic bacteria far outweighs the theoretical potential benefits. Milk-borne diseases cause unsurmountable economic losses, not to mention the resulting public health consequences. For example, in the United States of America (USA) and France, separate outbreaks of *E. coli* and *Salmonella* in retailed dairy products made from raw milk have caused national and international recalls. The concern about the safety of raw milk is more apparent when small, individual farms grow into larger, commercial-scale productions to meet the increased demand for milk. Since the prevalence of milk-borne *E. coli* and *Salmonella* in East Malaysia (Sarawak) has not been investigated, we aimed to: a) enumerate *E. coli* and *Salmonella* spp., and b) detect and evaluate the genetic-relatedness of Shiga toxin-producing *E. coli* and *S.* Typhimurium strains of the isolates.

STUDY OBJECTS AND METHODS

Sample collection. Samples of cow and goat raw milk were purchased from six farms in the southern region of Sarawak (Table 1). A total of 200 raw cow and goat milk samples were purchased over ten months, during 17 trips between April 2014 and January 2015. All the samples (50–200 mL per sample) were purchased in sterile plastic bottles and kept in an ice box to be transported to the Molecular Microbiology Laboratory at the University of Malaysia, Sarawak.

Enrichment and enumeration of *Escherichia coli* and *Salmonella* spp. by the MPN method. *E. coli* were isolated and enriched by the most probable number (MPN) method, as previously described with some modification [15]. For this, 10 mL of raw milk was transferred into a sterile Stomacher bag and mixed with 90 mL of Tryptone Soy Broth. The mixture was homogenized for 60 s and incubated at 37°C for 24 h. The enriched cultures were subjected to a three-tube MPN method. The MPN index was calculated based on 95% confidence limits for various combinations of positive tubes in a three-tube dilution series using 1, 0.1, and 0.01 mL for *E. coli* and *Salmonella* spp. detection in the samples. As indicated with tube turbidity, positive samples were cultured

Table 1 Raw milk samples from different dairy farms

Farm	Farm feature	Type of raw milk	Total number of samples
A	Small dairy goat and horse farm	Goat	65
B	Small dairy goat farm	Goat	6
C	Small dairy goat farm	Goat	17
D	Mixed dairy farm comprising cattle, goats, buffaloes, and horses	Goat	5
E	Small dairy goat farm	Goat	7
F	Large cow farm	Cow	100
			Total: 200

on EMB (Oxoid, USA) and XLD (Oxoid, USA) agars for *E. coli* and *Salmonella* spp., respectively. The grown colonies were picked and used in molecular analysis.

Genomic DNA extraction. Bacterial DNA was extracted using the boiling extraction method [16, 17]. For this, an aliquot of 1.5 mL of an overnight MPN suspension was centrifuged (Hettich EBA21 Zentrifugen, Germany) at 10 000 rpm for 5 min. Then, the suspension was boiled for 20 min and promptly chilled in ice for 20 min. Afterwards, it was centrifuged at 10 000 rpm for 5 min. The final supernatant containing bacterial DNA was collected and stored at – 20°C for further use.

Detection of Shiga toxin-producing *E. coli* and *E. coli* O157:H7. The Multiplex-PCR was conducted to detect *E. coli* strains in the raw milk samples using four primer pairs that target the *Stx1* and *Stx2* genes (encoding Shiga-like toxins 1 and 2), *rfbE* gene (encoding the somatic antigen, or O-antigen), and $fliC_{h7}$ (encoding the flagellar antigen, or H7-antigen) (Table 1) [18, 19]. The PCR was conducted using GoTaq® DNA polymerase (Promega, USA) with PCR conditions comprising a cycle of initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The DNA of *E. coli* O157:H7 was used as a positive control. The PCR product was analyzed by using agarose (1.5%) gel electrophoresis

Specific-PCR for detecting *Salmonella enterica* serovar Typhimurium. The Specific-PCR assay was carried out to detect *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). by using the *Fli15* and *Tym* primers that are specific to the *fliC* gene of *S.* Typhimurium, with minor modifications (Table 2) [20]. The PCR was conducted using GoTaq® DNA Polymerase (Promega, USA) with PCR conditions comprising a cycle of initial denaturation of 95°C for 5 min, 35 cycles of denaturation at 94°C at 60 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, and a cycle of final extension at 72°C for 1 min. The DNA of *S.* Typhimurium strain ATCC 14028 was used as a positive control. The PCR product was analyzed by using agarose (1.5%) gel electrophoresis.

BOX-PCR genotyping of *E. coli* and *S.* Typhimurium isolates. The BOX-PCR was conducted on *E. coli* and *S.* Typhimurium using a single BOXA1R primer: (5'-CTACGGCAAGGCGACGCTGACG-3'), as well as a TopTaq PCR Master Mix Kit (Qiagen, Germany) [8]. The PCR conditions comprised a cycle of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and a cycle of final extension at 72°C for 10 min. The PCR product was analyzed with the help of agarose (1%) gel electrophoresis.

ERIC-PCR genotyping of *E. coli* and *S.* Typhimurium isolates. The ERIC-PCR was conducted on *E. coli* and *S.* Typhimurium using the primers ERIC-1 (5'-ATG TAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'), as well as GoTaq® DNA Polymerase (Promega, USA) [8]. The PCR conditions comprised a cycle of initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 90°C for 30 s, annealing at 50°C for 1 min, extension at 72°C for 5 min, and a cycle of final extension at 72°C for 15 min. The PCR product was analyzed by agarose (1%) gel electrophoresis.

Phylogenetic data analysis. DNA fragments amplified in BOX-PCR and ERIX-PCR were analyzed for their electrophoretic profile [8]. RAPDistance and PyElph 1.3 gel analysis softwares were employed to determine the respective clonal relatedness of *E. coli* and *S.* Typhimurium. Normalization steps were included in the analysis to ensure adequate gel-to-gel banding pattern comparison. A band-scoring procedure identified bands in each lane that made a fingerprint based on the threshold of stringency and optimization settings. Utilizing PyElph 1.3 software, the positions of the marker run in BOX-PCR and ERIC-PCR were normalized from lane-to-lane and gel-to-gel variations. The unweighted pair group method with arithmetic mean (UPGMA) cluster analysis was performed in combination with the neighbor-joining tree (NJTREE) method and displayed in dendrograms.

The discriminatory index (*D*) of both BOX-PCR and ERIC-PCR was calculated based on Simpson's Diversity Index. A value of 0 (zero) indicates an identical pattern between isolates, whereas a value of 1 indicates a complete dissimilarity between isolates, corresponding to the higher Simpson's Diversity Index, the greater the discriminatory power of the typing tool [8].

$$D = 1 - \left(\frac{1}{N(N-1)} \right) \sum_{j=1}^{s} n_j (n_j - 1)$$

where *D* is the discriminatory index (DI); *N* is the total number of colonies in the sample population; *s* is the total number of clusters described; n_j is the number of colonies belonging to the cluster.

RESULTS AND DISCUSSION

Occurrence and concentration of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium in the fresh raw milk samples. We examined 200 samples of raw milk from six dairy farms, including 100 cow milk and 100 goat milk samples, for the presence of *E. coli* and *S.* Typhimurium. *E. coli* colonies appeared on EMB agar as dark blue-black growth with a green-metallic sheen, while *S.* Typhimurium colonies appeared on XLD agar in shiny and small-to-medium colorless shape after 24 h. Out of the total milk samples, 83.5% (167/200) and 65.5% (131/200) showed MPN values greater than 1100 MPN/mL for the presence of *E. coli* and *Salmonella* spp., respectively (Table 2).

Detection of Shiga toxin-producing *E. coli* and *S.* Typhimurium by PCR. Out of 200 raw milk samples tested, only 1.5% (3 samples) were found to be positive for *E. coli* O157:H7. However, a higher prevalence (9.5%; 19 samples) was found to be Shiga toxin-producing *E. coli* (STEC) of different serogroups which carried the

Table 2 Most probable numbers of *Escherichi coli* and *Salmonella* spp. in raw milk samples

Raw milk type	Bacteria	Number of samples	MPN/mL	95% confidence level	
				Lower	Upper
Goat milk	*E. coli*	1	20	0.45	4.2
Goat milk	*E. coli*	1	21	0.45	4.2
Goat milk	*E. coli*	1	28	0.87	9.4
Goat milk	*E. coli*	1	35	0.87	9.4
Goat milk	*E. coli*	2	36	0.87	9.4
Goat milk	*E. coli*	5	93	1.8	42
Goat milk	*E. coli*	5	150	3.7	42
Goat milk	*E. coli*	5	210	4.0	43
Goat milk	*E. coli*	1	240	4.2	100
Goat milk	*E. coli*	2	460	9.0	200
Goat milk	*E. coli*	9	1100	18	410
Goat milk	*E. coli*	67	**> 1100**	42	–
Goat milk	*Salmonella* spp.	1	21	0.45	4.2
Goat milk	*Salmonella* spp.	6	28	0.87	9.4
Goat milk	*Salmonella* spp.	5	36	0.87	9.4
Goat milk	*Salmonella* spp.	6	93	1.8	42
Goat milk	*Salmonella* spp.	6	150	3.7	42
Goat milk	*Salmonella* spp.	10	210	4.0	43
Goat milk	*Salmonella* spp.	6	240	4.2	100
Goat milk	*Salmonella* spp.	2	460	9.0	200
Goat milk	*Salmonella* spp.	15	1100	18	410
Goat milk	*Salmonella* spp.	43	**> 1100**	42	–
Cow milk	*E. coli*	100	**> 1100**	42	–
Cow milk	*Salmonella* spp.	3	150	3.7	42
Cow milk	*Salmonella* spp.	9	1100	18	410
Cow milk	*Salmonella* spp.	88	**> 1100**	42	–

The MPN/mL values in boldface indicate concentrations of the bacteria in the samples > 1100 MPN/mL

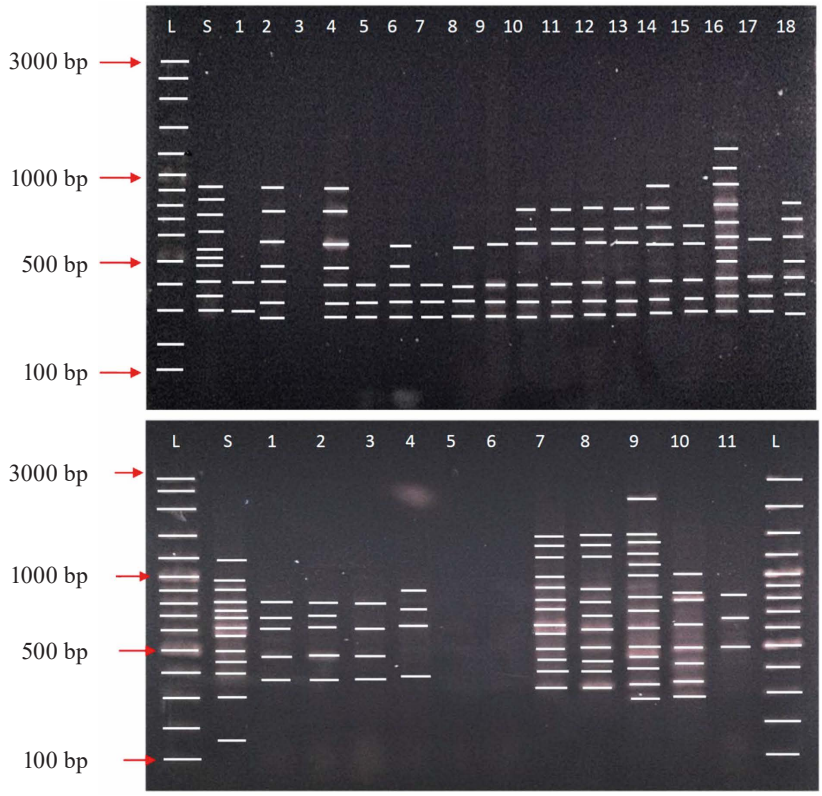
Table 3 Numbers of raw cow and goat milk samples positive for the targeted genes of *Escherichi coli* and *Salmonella enterica* serovar Typhimurium

Bacteria	Virulence genes	Cow milk	Goat milk	Serogroup	Total
E. coli	*Stx1/Stx2/rfbE+/fliC*$_{h7}$	1	0	STEC O157:H7	3/200 (1.5%)
	Stx2/rfbE+/fliC$_{h7}$	2	0		
	Stx2/fliC$_{h7}$	4	1	STEC of other serogroups	19/200 (9.5%)
	Stx2/rfbE+	14	0		
	Stx1/Stx2	0	0		
	Stx1	1	0		
	Stx2	2	1		
	rfbE+/fliC$_{h7}$	9	1	Non-STEC O157:H7	10/200 (5%)
	rfbE+	42	10	Non-STEC of other serogroups	83/200 (41.5%)
	fliC$_{h7}$	15	16		
S. Typhimurium	fliC	11	68	–	79/200 (39.5%)

Stx1 and/or *Stx2* genes but did not possess *rfbE+* or *fliC*$_{h7}$. Other than that, 5% (10 samples) and 41.5% (83 samples) were found to be non-STEC O157:H7 and non-STEC of other serogroups, respectively, because they lacked the *Stx* genes. Finally, 39.5% (79 samples) of the milk samples tested positive for *S.* Typhimurium (Table 3).

Virulence profiles of *E. coli* and *S.* Typhimurium. *E. coli* were classified into four groups based on the presence of Shiga-toxin genes (*Stx1* and *Stx2*) and the *rfbE* and *fliC*$_{h7}$ genes, namely STEC O157:H7, STEC of other serogroups, non-STEC O157:H7, and non-STEC. STEC indicates Shiga toxin-producing *E. coli*.

Genotyping of *E. coli* and *S.* Typhimurium using BOX-PCR and ERIC-PCR. The BOX-PCR genotyping for *E. coli* produced 2–11 bands for the raw cow milk samples (Fig. 1) and 3–14 bands for the raw goat milk samples (Figure not shown). Simpson's Diversity Index (SID) was utilized to measure the species diversity in a community. This index was adjusted to generate a numerical index for the discriminatory ability of single



E. coli isolated from Farm F (top). L: 100 bp ladder, Lane S: Standard, Lane 1: EC-CM83, Lane 2: EC-CM84, Lane 3: EC-CM85, Lane 4: EC-CM86, Lane 5: EC-CM87, Lane 6: EC-CM88, Lane 7: EC-CM89, Lane 8: EC-CM90, Lane 9: EC-CM91, Lane 10: EC-CM92, Lane 11: EC-CM93, Lane 12: EC-CM94, Lane 13: EC-CM95, Lane 14: EC-CM96, Lane 15: EC-CM97, Lane 16: EC-CM98, Lane 17: EC-CM99, Lane 18: EC-CM100. "EC" denotes *E. coli*, "CM" denotes cow milk sample

S. Typhimurium from Farm A (bottom). L: 100 bp ladder, Lane S: Standard, Lane 1: ST-CM3, Lane 2: ST-CM5, Lane 3: ST-CM56, Lane 4: ST-CM58, Lane 5: ST-CM59, Lane 6: ST-CM67, Lane 7: ST-CM70, Lane 8: ST-CM77, Lane 9: ST-CM78, Lane 10: ST-CM82, Lane 11: ST-CM83. "ST" denotes *Salmonella enterica* serovar Typhimurium, "CM" denotes cow milk sample

Figure 1 Gel electrophoresis of BOX-PCR

or combined typing systems. For raw cow milk, the SID among *E. coli* isolates was 0.989, which indicated an average genetic similarity of 40% between 100 isolates. For raw goat milk, the SID among *E. coli* isolates was 0.992, indicating an average genetic similarity of 45% between 40 isolates.

The BOX-PCR for *S.* Typhimurium in goat milk showed that only 9 out of 11 samples were successfully genotyped, producing 5–10 bands for raw cow milk (Fig. 1) and 4–14 bands for raw goat milk (Figure not shown). The SID among *S.* Typhimurium isolates was $D = 0.985$ for raw cow milk, indicating an average genetic similarity of 35% between 100 isolates. For raw goat milk, the SID was $D = 0.999$, indicating an average genetic similarity of 30% between 40 isolates.

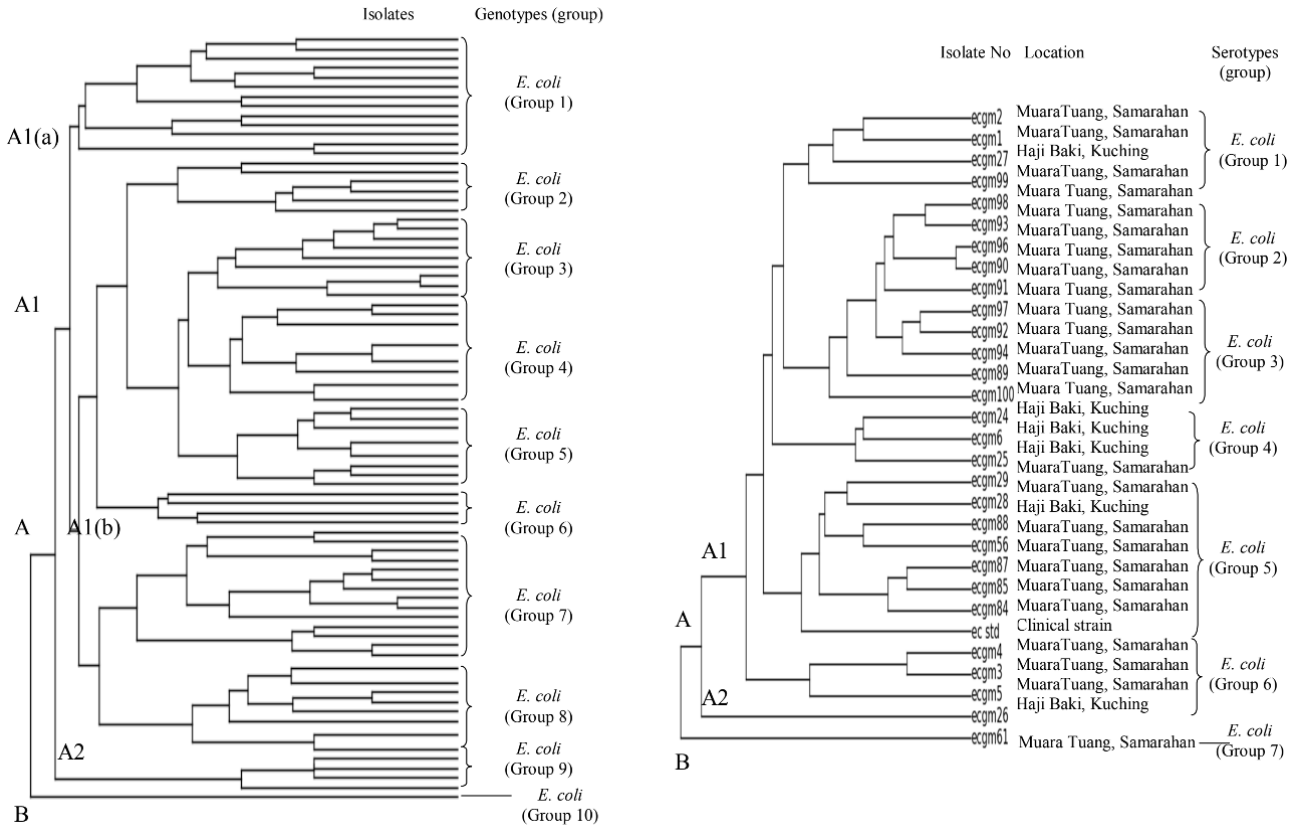
The dendrograms of the BOX-PCR for *E. coli* and *S.* Typhimurium isolates in both cow and goat milk were grouped into two clusters (A and B). Each of the clusters was further subdivided into several sub-clusters (Fig. 2). *E. coli* isolates were randomly grouped into different clusters and sub-clusters, indicating greater heterogeneity in the BOX-PCR DNA profiling compared to *S.* Typhimurium.

The ERIC-PCR for *E. coli* produced 2 to 13 bands in the raw cow milk samples (Fig. 3) and 2 to 11 bands in the raw goat milk samples (Figure not shown). Simpson's Diversity Index (SID) for *E. coli* isolates was $D = 0.997$ in the cow milk samples, indicating an average genetic similarity of 40% among 100 isolates, and $D = 0.980$ in the goat milk samples, indicating an average genetic similarity of 40% among 40 isolates.

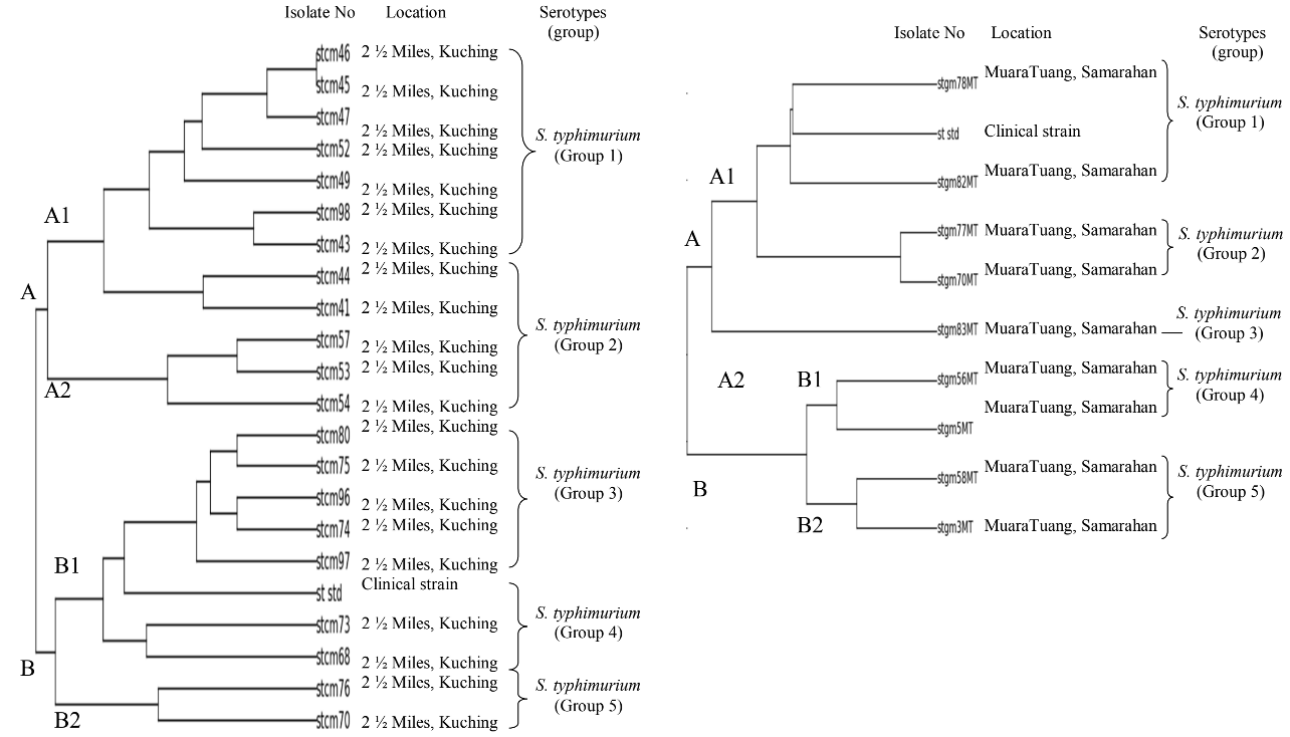
The ERIC-PCR for *S.* Typhimurium produced 3 to 14 bands in the raw cow milk samples (Fig. 3) and 2 to 4 bands in the raw goat milk samples (Figure not shown). The SID of *S.* Typhimurium isolates was $D = 0.900$ in raw cow milk, indicating a 35% genetic similarity between 100 isolates, and $D = 0.872$ in raw goat milk, indicating a 50% genetic similarity between 40 isolates.

Based on the dendrograms (Fig. 4), *E. coli* and *S.* Tymurium isolates were grouped into two clusters (A and B) for both cow and goat milk samples. *E. coli* isolates were randomly grouped into different clusters and subclusters. Despite sharing the same genotype from the same sampling sites, they showed more heterogeneity in the ERIC-PCR profiling compared to *S.* Typhimurium.

E. coli



S. Typhimurium



E. coli (top). The left side is the dendrogram for cow milk samples from Farm F (2 $^{1}/_{2}$ Miles, Kuching), while the right side is the dendrogram for goat milk samples from Farms A (Muara Tuang, Samarahan) and B (Haji Baki, Kuching)

S. Typhimurium (bottom). The left side is the dendrogram for cow milk samples from Farm F (2 $^{1}/_{2}$ Miles, Kuching), while the right side is the dendrogram for goat milk samples from Farm A (Muara Tuang, Samarahan)

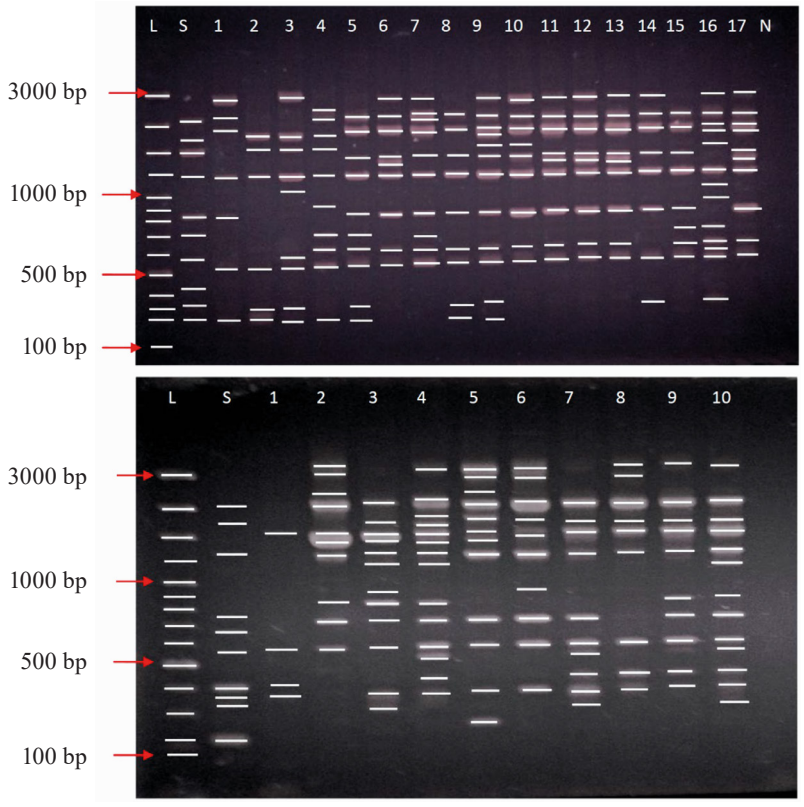
Figure 2 Dendrograms for BOX-PCR

The discriminatory power of genotyping tools is indicated by Simpson's Diversity Index (*D* value). In particular, the higher the *D* value, the greater the effectiveness of a particular fingerprinting method in strain discrimination. The *D* value of 1 represents maximum diversity where no isolates are similar. As shown in Table 4, the BOX-PCR demonstrated better discriminatory power (for both *E. coli* and *S.* Typhimurium) than the ERIC-PCR, with *D* values ranging from 0.985 to 0.999 (mean: 0.991) and from 0.872 to 0.997 (mean: 0.937), respectively.

Our study revealed high concentrations of *E. coli* and *Salmonella* spp. in both milks since large numbers of the samples had bacterial concentrations exceeding 1100 MPN/mL. In particular, out of all cow milk samples, 100 were positive for *E. coli* and 88 for *Salmonella* spp., while among the goat milk samples, 67 were positive for *E. coli* and 43 for *Salmonella* spp. The prevalence of high bacterial concentrations was found to be greater in cow milk (150 to > 1100 MPN/mL) than goat milk (20 to > 1100 MPN/mL).

In Malaysia, the permissible limits of total plate and coliform counts in pasteurized milk are 10^5 and 5×10 per mL, respectively, with no specified detection limit for *Salmonella*. In Australia and New Zealand, raw milk must undergo stringent controls and meet the microbial limits for *E. coli* (3 organisms/mL) and *Salmonella* (undetected in 25 mL). If retailed milk products exceed these limits, they must be recalled. While infectious concentrations of foodborne bacteria differ depending on their serovars, they are determined as 10–100 organisms for *E. coli* O157:H7 and 10^7–10^9 CFU/g for *Salmonella* [21]. However, given the lipid-dependent nature of infection, these concentrations can be lower in high-fat milk, with 1–5 cells potentially able to cause infection [22].

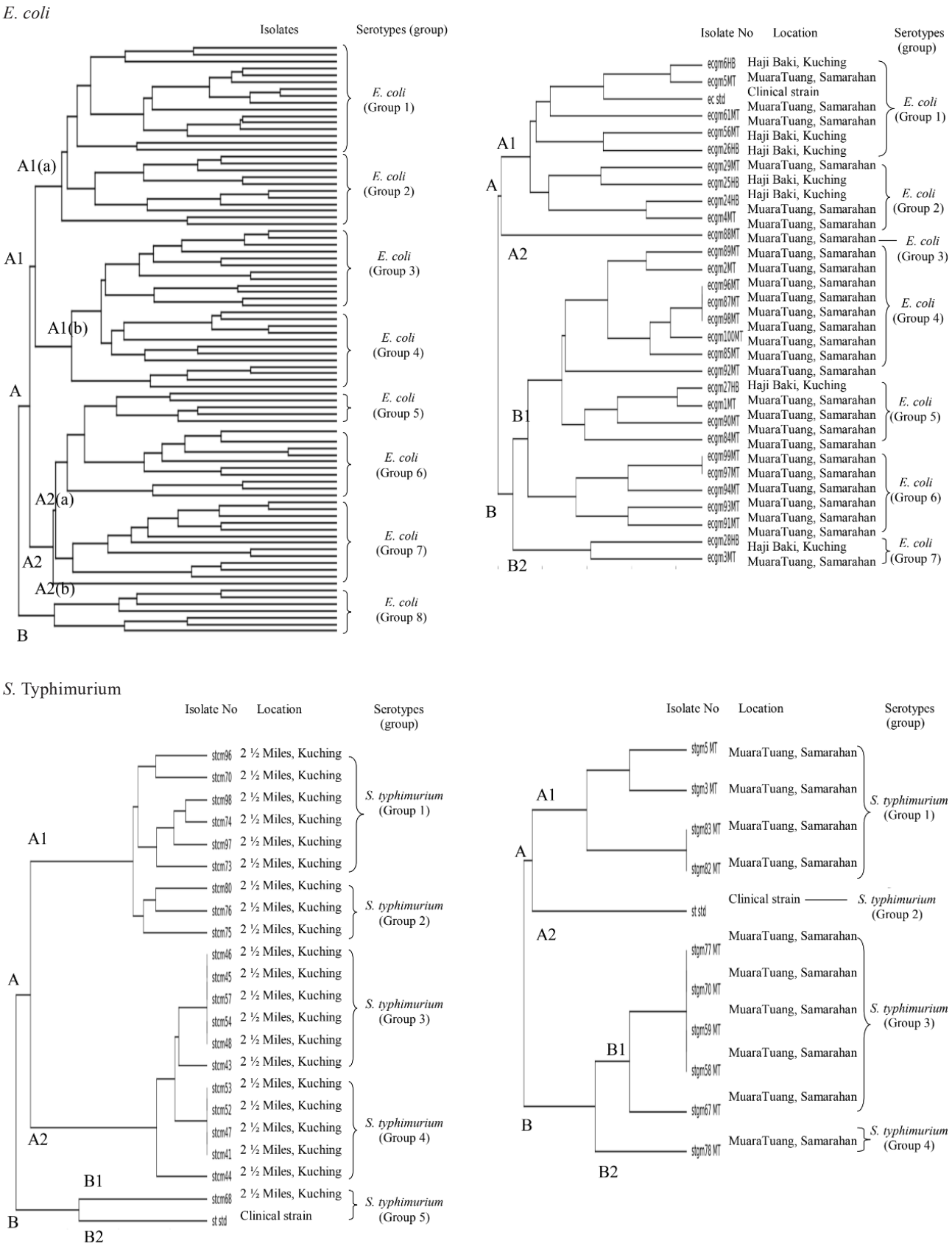
In this study, we could not rule out a possibility of contamination due to various factors contributing to milk spoilage. *E. coli* and *Salmonella* spp. may be introduced into milk directly from cow's blood (systemic infection), due to mastitis (udder infection) or cross-contamination among cows during milking, as well as from environmental sources (feces, water, pasture) [23]. However, these risks can be reduced by exercising good hygienic practices. Pathogenic *E. coli* and *Salmonella* spp. are among common bacterial contaminants in raw



E. coli from Farm F (top). L: 100 bp ladder, Lane S: Standard, Lane 1: EC-CM84, Lane 2: EC-CM85, Lane 3: EC-CM86, Lane 4: EC-CM87, Lane 5: EC-CM88, Lane 6: EC-CM89, Lane 7: EC-CM90, Lane 8: EC-CM91, Lane 9: EC-CM92, Lane 10: EC-CM93, Lane 11: EC-CM94, Lane 12: EC-CM95, Lane 13: EC-CM96, Lane 14: EC-CM97, Lane 15: EC-CM98, Lane 16: EC-CM99, Lane 17: EC-CM100, Lane N: Negative control. "EC" denotes *E. coli*, "CM" denotes cow milk sample

S. Typhimurium from Farm A (bottom). L: 100 bp ladder, Lane S: Standard, Lane 1: ST-CM68, Lane 2: ST-CM70, Lane 3: ST-CM73, Lane 4: ST-CM74, Lane 5: ST-CM75, Lane 6: ST-CM76, Lane 7: ST-CM80, Lane 8: ST-CM96, Lane 9: ST-CM97, Lane 10: ST-CM98. "ST" denotes *Salmonella enterica* serovar Typhimurium, "CM" denotes cow milk sample

Figure 3 Gel electrophoresis of ERIC-PCR



E. coli (top). The left side is the dendrogram for cow milk samples from Farm F (2 1/2 Miles, Kuching), while the right side is the dendrogram for goat milk samples from Farms A (Muara Tuang, Samarahan) and B (Haji Baki, Kuching)

S. Typhimurium (bottom). The left side is the dendrogram for cow milk samples from Farm F (2 1/2 Miles, Kuching), while the right side is the dendrogram for goat milk samples from Farm A (Muara Tuang, Samarahan)

Figure 4 Dendrograms of ERIC-PCR

Table 4 Summary of discriminatory power for ERIC-PCR and BOX-PCR

Genotyping method	Sample type	Bacteria	Number of types	Simpson's Diversity Index (*D* value)
BOX-PCR	Cow milk	*E. coli*	34	0.989
	Goat milk	*E. coli*	22	0.992
	Cow milk	*S.* Typhimurium	19	0.985
	Goat milk	*S.* Typhimurium	10	0.999
ERIC-PCR	Cow milk	*E. coli*	41	0.997
	Goat milk	*E. coli*	24	0.980
	Cow milk	*S.* Typhimurium	13	0.900
	Goat milk	*S.* Typhimurium	11	0.872

milk, as well as *Campylobacter* spp., *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Staphylococcus aureus* [24]. To prevent disease, it is important to ensure safe milk production from healthy animals. In addition, farm owners and milking workers should be trained to adhere to hygienic practices, and collected milk should be pasteurized immediately. However, this does not eliminate the risk of unsuccessful inactivation of *E. coli*, especially due to suboptimal pasteurization or post-contamination from milk-contact surfaces. Several outbreaks have been reported to be linked to pasteurized milks [25, 26]. Heat-resistant *E. coli* can refold and repair the denatured proteins by maintaining the integrity of cell envelopes and elevating the synthesis of heat shock proteins and chaperones after high temperatures [27]. Since *E. coli* can survive in processed pasteurized milk, and even grow faster due to less antagonistic interaction with pre-existing bacteria, processed milk should be stored at 5°C or below to avoid post-processing contamination [26, 28].

The higher prevalence of STEC in cow milk than in goat milk was due to cattle being a major reservoir of STEC [29]. Martin and Beutin found similar concentrations of STEC in the food products as in the original animal species, indicating that the contamination was likely to come from the animals rather than from humans or the environment [30]. Our study detected three *E. coli* O157:H7 isolates (1.5%; 3/200) in the cow milk samples (Farm F). Specific virulence factors such as Shiga-toxin and adherent fimbriae are linked to frequent cases of hemolytic uremic syndrome (HUS) and bloody diarrhea. O-antigen has high levels of chemical composition and variation structure, thus exhibiting different survivability and virulence across different strains [31]. The O serogrouping of *E. coli* strains provides important information for identifying pathogenic clonal groups. For example, O157 is a leading O serogroup associated with enterohemorrhagic *E. coli* (EHEC) and related to foodborne diseases worldwide. The most important strain detected in our study was *E. coli* O157:H7 that possessed a single *Stx2* gene, since this strain is more highly associated with causing hemolytic uremic syndrome than the strains that produce both *Stx1* and *Stx2* [29]. Although *E. coli* is inactivated at temperatures exceeding 63°C, the milk containing thermostable Shiga toxins can still pose health problems [32]. Even though a large proportion of the milk samples were not positive for STEC (Table 3), there remains concern if the other serogroups, which we did not analyze, could contain other important pathogens such as ETEC or EPEC.

S. Typhimurium, a contaminant that frequently degrades raw milk quality, was found in 39.5% (79/200) of the samples in our study. Similar to STEC, cow milk had a higher occurrence of *S.* Typhimurium than goat milk. Wang *et al.* detected *S.* Typhimurium in the PCR by targeting the flagellin gene, *fliC*, which encodes a major component of flagellum in the *S.* Typhimurium [33]. The flagellum of *S. enterica* is made up of a single protein called "flagellin", which consists of about 490 amino acids varying among the serovars. Flagellin is the main structural protein for flagella that is important during the initial stage of infection, involving mortality and invasion [34].

We analyzed the genetic relatedness of the *E. coli* strains isolated on the basis of the BOX-PCR fingerprinting patterns and found that the isolates were genetically heterogeneous, with average similarities of 40 and 45% in the cow and goat milk samples, respectively. The *S.* Typhimurium isolates showed genetic heterogeneity too, with average similarities of 30 and 35% in the cow and goat milk samples, respectively (Table 4). A previous study analyzed 211 strains of *E. coli* collected from dairy farms, calves, feces, pigs, primates, humans, and food products by the repetitive-element polymerase chain reaction using the BOXA1 primer. The similarity of 65% suggested that the BOX-PCR had good discriminatory power and was effective in clustering *E. coli* strains according to the sources [35].

The *E. coli* strains isolated on the basis of the ERIC-PCR fingerprinting patterns showed genetic heterogeneity among all the samples (Fig. 4). The *S.* Typhimurium isolates were genetically heterogeneous too, with average similarities of 35 and 50% in the cow and goat milk samples, respectively. A previous study, which examined *Salmonella* isolates from a variety of sources (humans, animals, food, and environment) using the ERIC primer set, found each of five serotypes clustered together, with a minimum similarity of 74% [36]. We determined that both genotyping tools (BOX-PCR and ERIC-PCR) can discriminate both bacteria species (*E. coli* and *S.* Typhimurium) with varying degrees of discriminatory power.

CONCLUSION

We examined the safety of milk from the dairy farms in Southern Sarawak, specifically looking at the presence of bacteria associated with Shiga toxin-producing *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). Our results showed that regular inspections and surveillance are necessary to minimize the risk of bacterial contamination in milk at the farms. Additionally, our study demons trated the usefulness of the BOX-PCR as a tool for genotyping *E. coli* and *S.* Typhimurium. This tool showed better discriminatory power than the ERIC-PCR as a fingerprinting method to discriminate different strains.

CONTRIBUTION

L. Maurice Bilung, A. Zulkharnain, K. Apun, and E.S. Radzi designed the research. E.S. Radzi conducted the experiment. E.S. Radzi and A.S. Tahar validated the data. L. Maurice Bilung, K. Apun, R. Ngui, and A.S. Tahar drafted the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ABBREVIATIONS

BOX-PCR: BOX repetitive sequence-polymerase chain reaction; DAEC: diffusely adherent *Escherichia coli*; EAEC: enteroaggregative *E. coli*; EHEC: enterohaemorrhagic *E. coli*, EIEC: enteroinvasive *E. coli*; EMB: Eosin Methylene Blue; EPEC: enteropathogenic *E. coli*; ERIC-PCR: enterobacterial repetitive intergenic consensus-polymerase chain reaction; ETEC: enterotoxigenic *E. coli*; HUS: hemolytic uremic syndrome; MPN: Most Probable Number; PCR: polymerase chain reaction; Shiga toxin-producing *E. coli*; SID: Simpson's Diversity Index; TSB: Tryptone Soy Broth; XLD: Xylose Lysine Deoxycholate.

DATA AVAILABILITY

All the data are available in this manuscript.

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