Isolation of Shiga toxin-producing *Escherichia coli* O157 and non-O157 from retail imported frozen beef marketed in Saudi Arabia using immunomagnetic separation and multiplex PCR

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Abstract

Introduction **Shiga toxin-producing *Escherichia coli* (STEC), particularly *E. coli* O157:H7, is a major contributor to foodborne outbreaks globally. Both *E. coli* O157 and non-O157 strains can lead to severe health issues, including hemolytic colitis and hemolytic uremic syndrome, which can result in kidney failure.**

Methods **Two hundred and one frozen beef samples were purchased from various supermarkets located in the Eastern Province of Saudi Arabia and subsequently enriched in tryptic soy broth (TSB). From the enriched samples in TSB, 1 mL portion was mixed with immunomagnetic beads (IMB) coated with specific antibodies targeting the *E. coli* O157 O antigen. The beads, which contained the captured bacteria, were then streaked onto CHROMagar O157 and Sorbitol MacConkey (SMAC) agar. The DNA extracted from these samples was examined using multiplex PCR to identify potential virulence gene markers, specifically *stx*-1, *stx*-2, and *eae*.**

Results **Of the 201 examined samples, 88 (43.8%) and 106 (52.7%) were positive for *E. coli* and produced colorless and mauve colonies on SMAC agar and CHROMagar O157, respectively. Out of 298 isolates in total, 174 isolates of *E. coli* were isolated with IMB enrichment. The highest detection rate of virulence gene markers was found among isolates that had been isolated using IMB enrichment, where 25 (8.4%), 2 (0.7%) and 12 (4%) isolates tested positive for *stx*1, *stx*2, and *eae* genes respectively. Among 42 isolates harboring potential virulence gene markers, 11 isolates were identified as *E. coli* O157 (stx_1^+/eae^+ or stx_2^+/eae^+). ERIC-PCR genotyping was able to determine the genetic relatedness among 42 isolates of *E. coli* O157 and *E. coli* non-O157 into 10 types with four identical related clusters and a genetic similarity rate above 90% homology from the identified isolates.**

Conclusions **The present study gives a clear perspective on STEC contamination in imported frozen beef marketed in Saudi Arabia. Because of the many possibilities of STEC contamination in imported frozen beef, further studies on the spread of STEC at various levels of imported frozen meat are needed on a long-term basis.**

Keywords **Frozen beef, immunomagnetic beads, multiplex PCR, Shiga toxin, ERIC, *E. coli* O157.**

Introduction

Escherichia coli is a gram-negative bacteria belonging to the Enterobacteriaceae family and the genus *Escherichia*. *E. coli* inhabits the human gut, warm-blooded animals and may be found in the surrounding environment. Those that are considered pathogenic are able to cause serious illness, morbidity and in some cases mortality.[1]

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There are different classes of pathogenic *E. coli*, known as *E. coli* pathotypes, which mainly have water and food origins.[2] One of the most dangerous pathotypes that require attention and has a history of outbreaks throughout the world is enterohemorrhagic *E. coli* (EHEC), particularly, the O157:H7 serotype.[3,4] After contaminated food is consumed, EHEC has a special characteristic; it can survive the stomach's acidity and adhere to the bowel mucosa. As soon as it has attacked the bowel mucosa, extracellular substances, as well as different types of Shiga toxins (Stx1 and Stx2), are secreted. Shiga toxins and other virulence factors, such as O157 plasmid and intimin protein, share the pathogenesis of *E. coli* O157.[3,5] Patients who suffer from *E. coli* O157 infections may develop hemolytic uremic syndrome (HUS), which can represent a triple threat since it causes acute kidney injury, microangiopathy and thrombocytopenia. It may also cause hemorrhagic colitis in some cases.[6-9]

Every year, an estimated 2.8 million people worldwide suffer acute illness from Shiga toxin-producing *E. coli* (STEC) infections, a significant risk to global health.[10] While serogroup O157 remains the most commonly diagnosed, cases linked to other STEC serogroups are rising due to better detection methods. Since cattle harbor both O157 and non-O157 STEC, reducing their prevalence in cattle herds could significantly mitigate human infections risk.[10] Routinely, laboratory identification of food microbes has been observed based on phenotypic methods of detecting, identifying and characterizing foodborne pathogens at the species level; however, this is time-consuming and has low sensitivity.[11] During the last two decades, a variety of new and advanced technologies have been employed due to the high demand for rapid detection that are sensitive and specific and are able to identify foodborne pathogens on genomic levels. An example was the introduction of immunomagnetic beads (IMB) followed by PCR-based methods. The IMB methodology is based on monoclonal antibodies which are utilized to enhance the isolation of *E. coli* O157 from other bacteria in the food mixture.[12] Enhancement levels mainly depend on microbial diversity, surveillance, outbreak detection, and infection control of foodborne pathogens.[12-14]

A range of studies have been conducted to understand the prevalence of STEC producing *E. coli* O157 contamination in meat and ready to eat food worldwide.[15-20] In Saudi Arabia, there are few published studies investigating the occurrence of STEC and *E. coli* O157 contamination of meat, milk, slaughterhouse settings and beef carcasses focusing on few local animal products,[21-24] with the exception of a recent study that reported the presence of *E. coli* O157 in imported beef meat from India and Brazil.[25]

The current study aimed to establish a swift method for detecting both *E. coli* O157 and non-O157 strains by utilizing IMBs that are coated with antibodies specific to the *E. coli* O157 O antigen. This approach was applied to samples that had undergone treatment to concentrate and isolate bacteria following the enrichment phase. Additionally, the study aimed to investigate the presence of major potential virulence genes among strains isolated from imported frozen beef using IMB separation. The study also focused on analyzing the clonal genetic relationships among the isolates based on the country of origin of the samples, using enterobacterial repetitive intergenic consensus (ERIC)-PCR. To our knowledge, this study represents the first comprehensive report on *E. coli* O157 and non-O157 strains isolated from imported frozen beef in our region.

Methods

Beef sample collection

A total of 201 imported frozen beef samples were purchased from supermarkets in Al-Khobar and Dammam cities in Saudi Arabia between March and May 2018 and examined for the presence of *E. coli* O157. Most of the chilled

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frozen beef sold in these supermarkets and food stores were imported from Australia, Brazil, India, and New Zealand. The purchased beef consisted of 40 samples from Australia (Bobby Veal), 58 samples from Brazil (Ground Beef), 75 samples from India (Bobby Veal) and 28 samples from New Zealand (Chuck Steak). The samples purchased were kept in cool containers and transported to the laboratory immediately for analysis.

Beef samples enrichment

Each 25 g of beef sample was inoculated into 225 mL of tryptic soy broth (TSB) in sterile stomacher plastic bag and then the sample was blended in a laboratory blender (Seward Stomacher 400 circulator, UK) for 2 min. The homogenized samples were incubated at 42°C for 6 hours. After 6 hours of incubation, a loop full of each enriched sample was cultured on Sorbitol MacConkey (SMAC) Agar (Oxoid, UK) and CHROMagar O157 (CHROMagar, Paris, France) and further incubated at 37°C for 24 hours.

Immunomagnetic beads (IMB) procedures

The process of concentrating suspected *E. coli* O157 samples enriched in TSB involved the use of commercially available magnetic beads that are coated with antibodies targeting the *E. coli* O157 O antigen (Denka Seiken, Japan). The preparation of these magnetic beads has been adapted from our method previously described elsewhere.[26] In short, 1 mL from each enriched TSB sample was added to 9 mL of TSB for a second round of enrichment, which was then incubated at 37°C for 24 hours. Following this, 1 mL from the second enrichment was transferred into a 1.5 mL microcentrifuge tube and combined with 15 µL of IMB specific to *E. coli* O157. The tubes containing the IMB were gently inverted and allowed to incubate at room temperature for 40 minutes. A magnetic concentrator rack was then utilized to separate the beads that had captured the bacteria from the enriched samples, followed by three washes with phosphate buffer saline (PBS). Finally, the aggregated bacteria on the beads were resuspended in 50 µL of PBS and plated onto SMAC agar and CHROMagar O157, then incubated at 37°C for 24 hours. The results of the presumptive isolated *E. coli* O157, both with and without IMB treatment, were then analyzed and compared.

Genomic DNA extraction

DNA extraction was conducted on all selected presumptive colonies isolated with and without IMB from SMAC agar and CHROMagar O157. All identified isolates were sub cultured in 5 mL Luria Bertani (LB) broth and incubated at 37°C for 24 hours. After incubation, 1.5 mL of overnight LB culture was transferred to microcentrifuge tubes to prepare the pellet after centrifugation at 10,000 xg for 2 min. Then, the obtained pellet was resuspended in nuclease-free water and boiled in a water bath adjusted at 100°C for 15 min. The boiled sample was cooled immediately and stored in a –20°C fridge for future use.

Multiplex PCR assay for virulence genes

All presumptive isolates of *E. coli* O157 isolated with and without IMB were further assayed via multiplex-PCR amplification of the *eae*, *stx*1, and *stx*2 gene markers. The positive control strain EDL933 of *E. coli* O157:H7 was used in each multiplex PCR for amplification of *stx*1, *stx*2, and *eae* genes. A 348 bp region of the *stx*1 gene (F-5'CAGTTAATG TGGTGGCGAAGG-3' and R-5'CACCAGACAATG TAACCGCTG-3'), a 584 bp region of the *stx*2 gene (F- ATCCTATTC CCGGGAGTTTACG' and R-5' GCGTCATCGTATACACAGGAGC-3'), and a 482 region of the *eae* gene (F-5'TCAATGCAGTTCCGTTATCAGTT-3' and R-5'GTAAAG TCCGTTACC CCAACCTG -3') were performed as outlined by previously published protocols.[27,28] Briefly, the total volume of the reaction mixture was 25 µL consisting of 12.5 µL of GoTaq Green Master Mix (Promega, USA), 2 µL of each forward and reverse primer, 4.5 µL nuclease-free water and 2 µL of the DNA template. The multiplex PCR reactions were performed in a thermal cycler (ESCO-Swift

MaxPro, Singapore) with the following conditions: pre-denaturation at 96°C for 4 min, followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 57°C for 20 seconds, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. Finally, products of multiplex-PCR amplification were separated using electrophoresis with 2% agarose gels setting the voltage at 80 V for 90 min. The amplicons were compared with 100 bp DNA ladder (Promega, USA) and the amplified fragments were visualized through a gel documentation system (G: Box, Syngene, UK).

ERIC-PCR DNA fingerprinting analysis

All 42 isolates of *E. coli* O157 and non-O157 confirmed by multiplex PCR were typed using enterobacterial repetitive intergenic consensus (ERIC)-PCR DNA fingerprinting with ERIC2 repetitive primer sequence 5'-AAGTAAGT GACTGGGGTGAGCG-3' in volume of 25 µL mixtures reaction as described elsewhere.[29-31] The obtained band of ERIC-PCR fingerprints were calculated using Dice coefficient with an optimization of 1% and a position tolerance of 1% using Gel-J software.[32]

Results

Immunomagnetic beads (IMB) separation of *E. coli* O157

The incidence of *E. coli* O157 in the various examined samples of imported frozen beef is presented in Table 1. In this investigation, frozen beef samples were initially identified as positive for *E. coli* O157 due to colorless and mauve colonies on SMAC agar and CHROMagar O157, respectively, with and without IMB enrichment treatment. Out of the 201 samples analyzed, those subjected to IMB enrichment yielded the highest number of positive results for *E. coli* O157, while the lowest was observed in samples that did not undergo IMB treatment. Notably, the highest isolation rates on CHROMagar O157 were found in samples from Australia (25, or 62.5%) and Brazil (43, or 74.1%), as detailed in Table 1. Among the 201 frozen beef samples analyzed without IMB enrichment, 33 (16.4%) tested positive on SMAC agar, while 88 (43.8%) were positive on CHROMagar O157. Following IMB enrichment, the number of positive samples increased significantly to 58 (28.9%) on SMAC agar and 106 (52.8%) on CHROMagar O157, as shown in Table 1 and 2. This study demonstrates that the use of IMB in the secondary enrichment process significantly improved the isolation and recovery rates of *E. coli* O157 on both SMAC agar and CHROMagar O157 medium. The findings underscore the importance of enrichment techniques in enhancing the detection of this pathogen in beef samples.

Multiplex PCR assay for virulence genes

The distribution and contamination rate of *E. coli* O157 in examined samples of imported frozen beef is shown in Table 3. Among 298 presumptive isolates of *E. coli* O157, 25 (8.4%) tested positive for the *stx*1 gene, 2 (0.7%) for the *stx*2 gene, and 12 (4%) for the *eae* gene, which were recovered from the samples treated with IMB enrichment. In contrast, the detection of virulence genes in samples without IMB treatment was notably lower, with only 12 (4%) and 2 (0.7%) positive for *stx*1 and *eae* genes, respectively (Table 3). The highest prevalence of the *stx*1 gene was observed in isolates from imported frozen beef samples originating from Brazil and India (Table 3). The overall frequency distribution of virulence genes among the 298 isolates revealed that 12.4% were positive for the *stx*1 gene, while 4.7% were positive for the *eae* gene (Table 4). Notably, 11 isolates were classified as *E. coli* O157, exhibiting either a combination of virulence markers (*stx*1+/*eae*+ or *stx*2+/eae+) or solely stx1 or *stx*2, with the latter being categorized as *E. coli* non-O157. The prevalence rates for *E. coli* O157 and *E. coli* non-O157 in the examined frozen beef samples were found to be 3.7% and 10.4%, respectively (Table 5). The highest prevalence rates of *E. coli* non-O157 were recorded at 12% and 25.7% in imported frozen beef from India and Australia, respectively (Table 5).

ERIC-PCR typing

ERIC-PCR primer generated different DNA polymorphisms among 42 isolates revealing

Table 1. *E. coli* O157 in imported frozen beef

Country of origin	No. of samples	Number of positive samples (%)			
		Isolated without IMB on		Isolated with IMB on	
		SMAC Agar	CHROMagar O157	SMAC Agar	CHROMagar O157
India	75	24 (32)	17 (22.7)	25 (33.3)	21 (28)
Australia	40	8 (20)	24 (60)	13 (32.5)	25 (62.5)
Brazil	58	1 (1.7)	39 (67.2)	20 (34.5)	43 (74.1)
New Zealand	28	0	8 (28.6)	0	17 (60.7)
Total	201	33 (16.4)	88 (43.8)	58 (28.9)	106 (52.7)

Table 2. Isolation of *E. coli* O157 with and without IMB

Country of origin	Sample number	Number of isolates				Total
		Isolated without IMB on		Isolated with IMB on		
		SMAC Agar	CHROMagar O157	SMAC Agar	CHROMagar O157	
India	75	24	18	26	23	91
Australia	40	9	24	14	27	74
Brazil	58	2	39	23	44	108
New Zealand	28	0	8	0	17	25
Total	201	35	89	63	111	298

Table 3. Confirmation of *E. coli* O157 using multiplex-PCR assay

Country of origin	Number of tested isolates	Number of isolates positive for virulence gene					
		Isolated without IMB			Isolated with IMB		
		stx1	*stx2*	*eae*	*stx1*	*stx2*	*eae*
India	91	3	0	2	8	0	5
Australia	74	8	0	0	13	0	2
Brazil	108	1	0	0	4	1	4
New Zealand	25	0	0	0	0	1	1
Total (%)	298	12 (4%)	0	2 (0.7%)	25 (8.4%)	2 (0.7%)	12 (4%)

Table 4. Distribution of virulence genes among *E. coli* isolated from frozen beef (n=298)

Virulence gene profile	Number of isolates	Frequency of isolates (%)
*stx*1	37	12.4
*stxt*2	2	0.7
eae	14	4.7
*stx*1, *eae*	9	3
*stx*2, *eae*	2	0.7
*stx*1, *stx*2	0	0
*stx*1, *stx*2, *eae*	0	0

Table 5. Prevalence of O157 and non-O157 Shiga toxin producing *E. coli* isolated from frozen beef

Country of origin	Number of tested isolates	Number of positive isolates (%) for		Total
		E. coli O157	*E. coli* non-O157	
India	91	4 (4.4)	11 (12)	14 (15.4)
Australia	74	2 (2.7)	19 (25.7)	21 (28.4)
Brazil	108	4 (3.7)	2 (1.9)	6 (5.6)
New Zealand	25	1 (4)	0	1 (4)
Total	298	11 (3.7)	31 (10.4)	42 (14.1)

different bands ranging between 150 to 5000 bp (Figure 1). This technique successfully categorized all 42 isolates of *E. coli* O157 and non-O157 into 10 distinct genotypes using the ERIC2 primer. The analysis led to the formation of four clusters (A, B, C, and D) through the UPGMA algorithm and Gel J software. Among the isolates, 36 exhibited a genetic similarity exceeding the 90% threshold, while 6 isolates were identified as single lineages, indicating they were genetically distinct. Notably, ERIC-PCR effectively organized the isolates based on their country of origin, except for one non-O157 isolate (code 26B-3) from Australian beef, which was placed in cluster C and shared 100% genetic similarity with two isolates from Indian beef. Additionally, another non-O157 isolate (code 46A-3) from Brazilian beef was classified in cluster B, showing 95% genetic similarity with isolates from Australian beef.

Discussion

STEC infections are believed to result in approximately 2.8 million instances of serious illness each year across the globe.[10] Hemorrhagic colitis and HUS in humans are associated with the intake of contaminated beef products harboring STEC. Among foodborne illnesses, STEC-O157:H7 stands out as a primary contributor to serious outbreaks, particularly in regions with high consumption of beef. While serogroup O157 remains the most commonly detected STEC in human cases, there has been a notable increase in infections linked to non-O157 STEC serogroups, driven by improved surveillance methods and advancements in detection technologies.[10] Cattle serve as a major source for both STEC O157 and potentially non-O157 STEC, so reducing their prevalence in these animals could decrease the likelihood of human infections. The key virulence factors of STEC include the production of one or both Shiga toxins, known as *stx*1 and *stx*2. Moreover, the presence of either or both the *stx* and eae genes is regarded as a significant and heightened virulence characteristic of these pathogens.[33] The present study was undertaken to investigate the contamination of STEC-O157 and non-O157 in imported frozen beef in Saudi Arabia through the application of immunomagnetic separation combined with multiplex-PCR.

In this study, the overall reported strains harboring *stx*1 gene were isolated from examined frozen beef imported from India and Australia and the overall frequencies of *stx*1 gene among the 298 isolates were 12.7%. Whereas the overall frequency rate of *stx*2 gene was 0.7% and detected in two strains isolated from beef imported from Brazil and New Zealand. Additionally, the distribution rate of eae^+ gene among the overall isolates was 4.7% and reported in 6, 4, 3, and 1 strains isolated from examined frozen beef imported from India, Brazil, Australia and New Zealand, respectively. Therefore, the isolates that tested positive for the combined presence of $stx1^+$ and eae^+ or $stx2^+$ and eae^+ reported and categorized as *E. coli* O157 with prevalence rate of 3.7%, whereas those isolates reported positive for $stx1^+$ or $stx2^+$ gene regardless of absence of *eae* gene were reported as *E. coli* non-O157 with prevalence rate 10.4%. Furthermore, *E. coli* non-O157 was detected in examined frozen beef imported from Australia, India and Brazil, but none from New Zealand. In this study, the isolation of STEC in the examined imported frozen beef samples revealed 26 identified strains and is considered as a high isolation level of $stx1^+$ in non-O157. A similar study was conducted in the Southern region of Thailand were reported as having higher levels of $stx1^+$ non-O157 detection among examined marketed beef and reported large number of isolates carrying *stx*1 gene.[26,34] Our research aligns closely with a recent investigation carried out in 2017 by the Saudi Food and Drug Authority (SFDA), which identified *E. coli* O157:H7 in 6.8% of beef samples imported from India and 2.2% from Brazil. This correlation underscores the ongoing concerns regarding food safety concerning imported meat products.[25]

In the present study, the IMB was used via an enrichment treatment technique for detection of *E. coli* O157 from imported frozen beef samples. Enrichment using IMB conjugated with coated antibodies in beads against *E. coli* O157 antigen after pre-enrichment yielded a better detection on

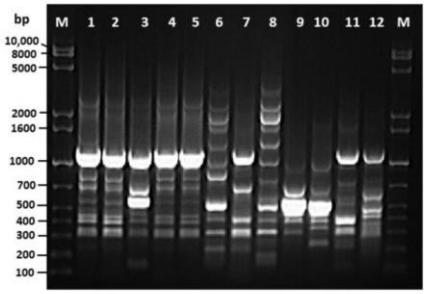

Figure 1. Representation of the ERIC DNA fingerprints of isolates and electrophoresed on 2% agarose gel

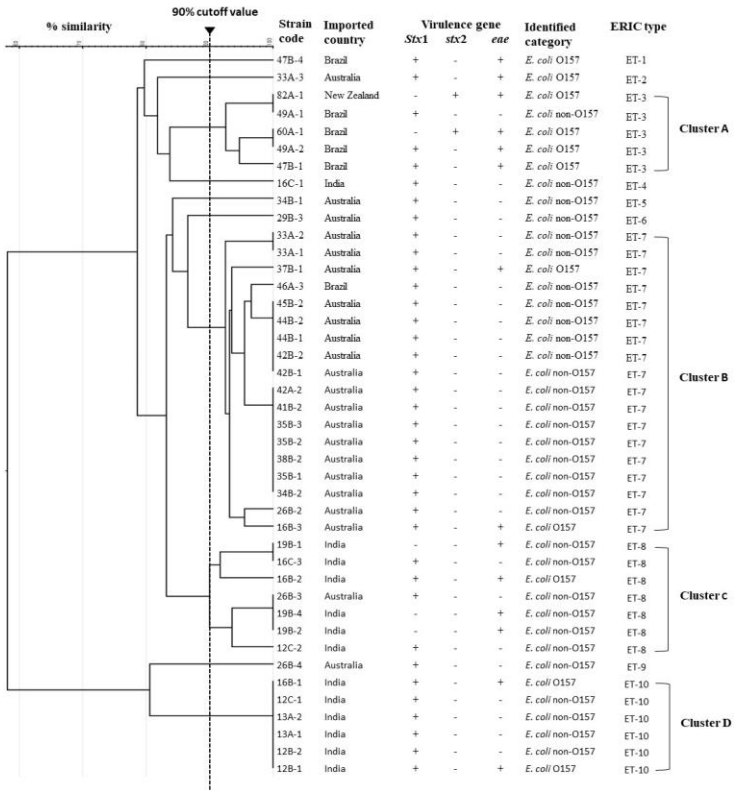

Figure 2. Cluster of ERIC-PCR fingerprints of 42 isolates, grouped using the UPGMA algorithm

SMAC agar and CHROMagar O157 medium. Subsequently, all examined beef samples were plated on similar selective culture mediums after pre-enrichment without IMB revealing lower detection. Therefore, the best isolation was observed when using IMB enrichment and CHROMagar O157 medium as a selective medium for isolation of *E. coli* O157 from frozen beef samples. Several published studies reported that application of IMB to the enrichment process after sample pre-enrichment, will modify the protocol making it more rapid and sensitive for separation and detection of *E. coli* O157 in comparison to the conventional culturable method.[35,36] In this study, the examined samples were pre-enriched for six hours and incubated at 42°C before the use of IMB separation. Hence, it is noteworthy to mention that the six-hour pre-enrichment has been reported to be more sensitive and effective rather than just one enrichment process for 24 hours.

Despite a variety of available techniques in molecular biology, for instance, random amplified polymorphism deoxyribonucleic acid, amplified fragment length polymorphism, pulsed field gel electrophoresis (PFGE), microarray and restriction fragment length polymorphism, the techniques propose a number of challenges including the requirement of high budget.[37-39] From published literature, several studies used ERIC-PCR fingerprints to type bacterial strains isolated from clinical, food and environmental samples, and was found to be cost-effective and faster than other typing techniques such as PFGE.[40] Among PCR based typing techniques, ERIC-PCR has frequently been used and proven a strong discriminatory power for a wide range of organisms in the *Enterobacteriaceae* family, such as *E. coli*.[41] In this study, ERIC-PCR was able to type all 42 isolates of *E. coli* O157 and non-O157 into four clusters (A, B, C and D), where 36 isolates were genetically related considering the genetic similarity value (>90% cut-off). On the other hand, 6 out of 42 isolates showed single lineages ERIC type (ET) patterns [ET-1, ET-2, ET-4, ET-5, ET-6 and ET-9] below 90% genetic similarity. These were considered genetically unrelated in accordance with a study published by Szczuka & Kaznowski (2004).[42] In this study, ERIC-PCR demonstrated that these strains were genetically related and were able to group the isolated strains into distinctive clusters according to their imported country of origin based on ERIC DNA fingerprints. Among the phylogeny of four clusters of ERIC-PCR constructed by UPGMA, cluster-B comprised the highest number of *E. coli* O157 and non-O157 isolated from frozen beef imported from Australia and, interestingly, one strain of *E. coli* non-O157 (46-A3) isolated from frozen beef imported from Brazil shared 96% genetic similarity with these strains in this cluster. On the other hand, one strain (82A-1) of *E. coli* O157 (*stx2*$^+$/*eae*$^+$) in cluster-A was isolated from imported frozen beef imported from New Zealand shared a 100% genetic similarity with strain (60A-1) of *E. coli* O157 (*stx2*$^+$/*eae*$^+$) isolated from imported beef imported from Brazil. Moreover, cluster-C and D comprised 13 strains of *E. coli* O157 and non-O157, 12 strains were isolated from frozen beef imported from India and one strain (26B-3) was isolated from frozen beef imported from Australia sharing a 100% genetic similarity. Therefore, the results reported in this study are in concordance with our previous published study which evaluated ERIC-PCR method for determining genetic diversity among *E. coli* isolated from human and retail imported frozen shrimp and beef, when the study concluded that ERIC-PCR is a useful molecular typing tool to discriminate pathogenic *E. coli*.[30]

Conclusions

The increasing reports of food contamination and outbreaks linked to foodborne illnesses highlight the pressing need for a quick and straightforward method to detect *E. coli* O157 in frozen imported beef and other meat products. This study showcased the effectiveness of IMB in secondary enrichment, which significantly improves the detection of *E. coli* O157, while also allowing for the confirmation of isolates through multiplex-PCR that targets essential virulence gene markers (*stx*1, *stx*2, and *eae*). The advantage of employing IMB enrichment in conjunction with the multiplex-PCR assay lies in its ability to swiftly identify *E. coli* O157 and screen for non-O157 STEC in food samples. This innovative approach could serve as a crucial rapid screening tool for inspecting imported frozen beef, thereby aiding in the prevention and control of STEC infections and contributing valuable insights to the realm of food safety.

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Conflicts of interest: All authors – none to declare.

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