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Original Research Article

Understanding the occurrence of polymerase chain reactions-positive and culture-negative for Shiga toxin producing *Escherichia coli* in samples from beef production chain

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ABSTRACT

Molecular epidemiological methods have been employed to detect pathogens and transmission pathways, for disease surveillance, outbreak investigation, outbreak monitoring and control. Molecular methods such as polymerase chain reactions (PCR) is used to assess the positivity rate of virulent gene(s) with pathogens, but in many cases, cultural isolation of the pathogen may not be possible in PCR positive cases. This dichotomy between the outcome of results may be associated with low number of cells compared with the large population of background microflora, presence of viable but non-culturable cells, loss of virulence gene (s) after subculture, and the high sensitivity of the PCR assay. Shiga-toxigenic *Escherichia coli* (STEC) was used as a model for investigating this phenomenon. In this study, duplex PCR was used to screen 335 abattoir and 303 beef retail outlets selective broth enriched samples for the presence of *stx1* and *stx2* genes. Subsequent culture isolation of *stx*-positive broth samples was carried out. The overall STEC positivity determined by PCR in 335 and 303 abattoir and beef retail outlets in selective enrichment broth samples, respectively was 35.2% (118/335; 95% CI: 30.1 - 40.6) and 12.5% (38/303; 95% CI: 9 - 16.8). Only 24 (20%; 24/118) abattoir and 8 (21%; 8/38) retail outlet *stx*-positive samples were culturable. Both yielded only 51 isolates; 30 isolates for abattoir samples and 21 isolates for beef retail outlets, respectively. Our results confirm the dichotomy of PCR positive/culture negative samples, and from an epidemiological perspective, it is recommended that the use of only PCR to detect virulence genes in broth cultures should be acceptable where isolation is not achievable. This may be the best method for generating relevant epidemiologic data for disease control.

Keywords: Cattle, PCR, Shiga-toxigenic *Escherichia coli*, *Stx* phages.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) has emerged as an important foodborne pathogen globally with significant impact on public health. Foods of cattle origin have been implicated in various foodborne and waterborne outbreaks and epidemiological investigations have revealed that cattle are a major reservoir of STEC (Karmali *et al.,* 2010).

Not all STEC strains are pathogenic (Hughes *et al.,* 2006), hence the need for virulence characterization to determine pathogenicity. The pathogenicity of STEC is controlled by a number of virulence factors that are encoded by chromosomal pathogenicity islands, phage chromosomes integrated in the bacterial genome as well as plasmids (Kaper, 1998). The essential virulence factor of STEC is a potent cytotoxin recognized as Shiga toxin (*stx*) or verocytotoxin (VT). Mobile genetic elements, such as plasmids, transposons, bacteriophages, genomic islands and insertion sequence elements can be acquired by lateral gene transfer, giving rise to high level of diversity and hybrid pathotypes (Karch *et al.,* 2012; Melton-Celsa *et al.,* 2011). Interestingly, many of these STEC virulence factors reside on mobile genetic elements and can be lost or transferred, thereby increasing the unpredictability of a true pathogenic STEC strain, because a new hybrid-pathotype can evolve as a result of the mobility of virulence genes (Karch *et al.,* 2012; Melton-Celsa *et al.,* 2011).

Accordingly, culture-based methods remain the gold standard test in the laboratory (Giuliano et al., 2019), given the epidemiological importance of subtyping viable bacterial isolates. Routine laboratory detection methods in samples include selective broth enrichment, followed by molecular-based detection method such as polymerase chain reaction

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(PCR) for *stx* genes, and subsequent cultural isolation for downstream analysis (Bacteriological Analytical Manual protocol for *Diarrheagenic Escherichia coli* (2022) US-FDA). However, laboratory results present a dichotomy between PCR positive and subsequent negative culture results. This discrepancy is reported across a wide spectrum of sample matrices including environmental, food and clinical samples. This phenomenon has been reported by researchers in PCR-based STEC detection studies in Europe (Ciupescu, Nicorescu, Mihai, Dumitrache, and Tanasuica, 2015), the U.S.A. (Woo and Palavecino, 2013), Argentina (Brusa *et al*., 2017) and South Africa (Kalule, Keddy, and Nicol, 2018). Furthermore, literature have suggested these significant discrepancies may be related to factors such as low concentration of the cells (Pradel *et al*., 2000; Samadpour *et al*., 2006), presence of viable but non-culturable cells (VBNC), cell stress or sublethal cell injury (Farrokh *et al*., 2013; Hoang Minh *et al*., 2015; Li *et al*., 2014; 2020) and the high sensitivity of the PCR assay, which can detect free *stx*- carrying phages sample matrices (Pradel *et al*., 2000; Samadpour *et al*., 2006). This study aims to investigate this dichotomy using standard laboratory methods of screening for STEC virulent genes and to summarize associated factors that may lead to this dichotomy.

MATERIALS AND METHOD

SAMPLE COLLECTION

The samples used in this study were obtained from two sources: beef abattoirs (faeces, carcass swabs and abattoir liquid waste) and beef retail outlets (Dried cured beef, ground beef and brisket flat cuts). During the period, November 2015 to November 2016, a random cross-sectional survey investigated STEC positivity in beef carcasses and beef products in Gauteng Province., South Africa. For the abattoir, the 335 samples used in this study comprised of carcass swabs in swab rinse kit solution (SRK), faeces, and abattoir liquid waste (affluent). For the beef retail, 303 samples were obtained by purchasing three different beef and beef products. The samples comprised brisket flat cuts, ground beef and ready to eat (RTE) dried cured beef. See table I.

A minimum of 50 g of each product was purchased from the outlets as sold to the buyer and transported to the laboratory ice-cooled. At the abattoir, pre-slaughter faecal samples were collected via rectal grab with a rectal glove at the lairage. The faecal samples were transferred into a 100 mL sterile specimen container. Following hide removal, composite swab samples from the rump, flank, brisket and neck areas were collected at different stages of processing and samples obtained at different locations in the processing plants using swab rinse kit (SRK) (Copan Diagnostics, Inc., UK) according to the European Union Decision 2001/471/EC.

LABORATORY ANALYSES: SAMPLE PREPARATION, ENRICHMENT AND PROCESSING

PREPARATION OF BROTH CULTURES FROM FAECAL MATERIALS

Twenty-five grams (25 g) of each faecal sample was aseptically weighed into a sterile stomacher bag (Seward, UK), to which 225 mL of Buffered Peptone Water modified with Pyruvate (mBPWp) (HiMedia laboratories, India) was added and the sample mixture homogenized in a Stomacher (Steward 400 Laboratory, UK). Homogenates were incubated at 37°C ± 1°C static for 5 h, after which 1mL of the Acriflavin 10mg - Cefsulodin 10 mg -Vancomycin 8 mg (ACV) Supplement (HiMedia laboratories, India) was added and incubated at 42°C ± 1°C static overnight (18-24 h). All broth enrichment procedures followed the Bacteriological Analytical Manual (BAM) protocol for Diarrheagenic *Escherichia coli* (2022) US-FDA with slight modifications.

PREPARATION OF SWAB SAMPLES AND ABATTOIR LIQUID WASTE

Abattoir liquid waste was aseptically centrifuged at 10,000 × g for 10 min (Beckman Coulter, AllegraTM X-22 Series, Germany) in graduated plastic 50 mL centrifuge tubes (Sigma Aldrich, Germany). The supernatant was decanted and the sediments/pellets were re-suspended in 225 mL of mBPWp (HiMedia Laboratories, India) into a sterile Seward 400 Laboratory Stomacher bag (Seward, UK). Homogenates were processed using the procedure described above for faecal samples. For swab samples, 10 mL of sample solution was added to 90 mL of Buffered Peptone Water modified with pyruvate (HiMedia Laboratories, India), in a Seward Stomacher bag (Seward, UK), mixed and processed using the procedure earlier described above for faecal samples).

PREPARATION OF BEEF SAMPLES

Each beef sample (25 g) was aseptically weighed into a sterile Seward 400 Laboratory Stomacher bag (Seward, UK), to which 225 mL of Buffered Peptone Water (BPW) modified with Pyruvate (mBPWp) (HiMedia Laboratories, Mumbai, India), was added and the sample was properly homogenized. Homogenates were processed using the procedure described above for faecal samples.

DETECTION OF STEC VIRULENCE GENES

In the extraction of DNA template from broth cultures, 1 mL of inoculated enrichment broth incubated overnight was transferred into a 1.5 mL sterile centrifuge tube for DNA extraction using Quick-gDNA™ MiniPrep Kit (Zymo-Research Irvine, CA) according to the manufacturer's instructions. The DNA extracts were stored at -20°C and subsequently used for screening by PCR.

Extracted DNA samples were investigated for the presence of *stx*1 and *stx*2 genes using a duplex PCR (dPCR) method as described by Paton and Paton (1998). The published

oligonucleotide primers *stx1* and *stx2*, were commercially synthesized (Inqaba Biotechnologies, Pretoria, South Africa) and reconstituted to 100 µM. Duplex PCR was conducted in a 25 µL volume containing 2-4 µL nucleic acid template (approximately 30-50 ng/µL DNA), 12.5 µL Thermo Scientific™ DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, USA) and 0.25 µM of each primer. The primer sequences, amplicon sizes, dPCR thermal cycles programme and the electrophoresis gel procedures were carried out as previously described by Paton and Paton (1998). The primers and the base pairs of PCR amplification products used in the study are shown in Table II and gel representative picture shown in Figure 1.
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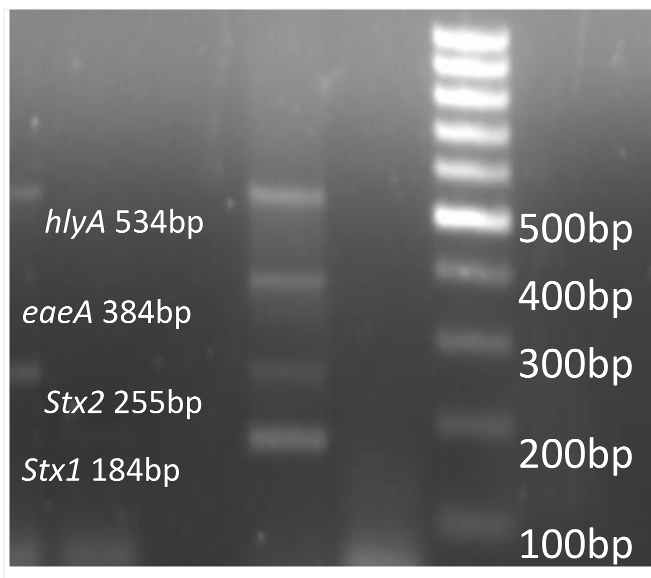

Figure 1. Representative gel picture with the expected product sizes

VALIDATION OF PCR

The assay conditions were optimized using molecular control strains obtained from NICD-CED, Johannesburg, South Africa (2014-2015 VTEC EQA—*E. coli* RR18-3022 O157, *eaeA*, *stx1a*, *stx2a*) and the enrichment control strain *E. coli* ATCC 43888 (O157:H7) *stx1*. The dPCR was validated by Sanger sequencing of PCR products. Only broth enrichments that had *stx1* and/or *stx2* were considered positive for STEC.

ISOLATION OF STEC STRAINS

Only enrichment broth cultures that were PCR-positive for *stx1* or *stx2* or both were considered positive for STEC and cultured to isolate STEC strains. To isolate O157 STEC the procedure consisted of immunomagnetic separation (IMS) assays using Dynabeads® anti-*E. coli* O157 (Thermo Fisher Scientific, USA), as recommended by the manufacturer. The immune-concentrated bacterial suspensions were then inoculated onto sorbitol with MacConkey agar (SMAC) supplemented with potassium tellurite 2.5 mg/L and cefixime 0.05 mg/L (Himedia Laboratories Pvt. India). Likewise, 10 µl of enriched broth sample was streaked on a chromogenic agar, CHROMagar O157 (CHROMagar Microbiology, Paris, France) supplemented with potassium tellurite 2.5 mg/L and cefixime 0.05 mg/L (Himedia Laboratories Pvt. India). Subsequently, the plates were incubated for 24-30 h at 37C. About 25 colonies that exhibited mauve colour phenotypic characteristics were selected from each plate and tested by latex agglutination (Welcolex® *E. coli* O157 Rapid latex agglutination test, Remel, UK). Enriched Control Strain –*E. coli* ATCC 43888 (O157:H7) was also inoculated for phenotypic control and assessment. Thereafter, presumptive isolates were confirmed by dPCR as described above.

To isolate non-O157 STEC, 10 µL of each enriched broth sample was streaked on MacConkey agar containing crystal violet and bile salt, and onto CHROMagar STEC (CHROMagar Microbiology, Paris, France). The plates were incubated for 24-30 h and about 25 representative suspect colonies were streaked on nutrient agar plates for further biochemical testing using Kovacs' indole reagent (Remel, California, USA). Indole positive isolates were confirmed with dPCR, as described earlier. In addition, biochemical confirmation as *Escherichia coli* using the bioMérieux Vitek 2 Compact system (bioMerieux, Marcy l'Etolie, France) was performed.

PREPARATION OF DNA TEMPLATE FOR PRESUMPTIVE STEC ISOLATES USING BOILING METHOD

Briefly, 10 µL disposable sterile loop (Whitehead Scientific (Pty) SA) was used to obtain a loop-full of pure culture from nutrient agar plates (Quantum Biotechnologies (Pty), South Africa). The culture was suspended in 1 mL molecular grade water, vortexed until thoroughly mixed and centrifuged for 10 min at 10,000g. The supernatant was decanted and 200 mL Tris-EDTA buffer (Inqaba Biotechnical Industries (Pty) SA) was added and vortexed until suspension was properly mixed. The suspension was boiled on a heating block (Merck, New Jersey, USA) at 95C for 15 min and allowed to cool at room temperature. The supernatant was transferred to a sterile 1.5 mL centrifuge tube and stored at -20C for use.

Saline was used as a negative control while 2014-2015 STEC EQA—*E. coli* RR18-3022 O157 (*eae*, *stx1a*, *stx2a*) was used as positive control.

STATISTICAL ANALYSIS

Duplex PCR results of the broth cultures of samples were used for this analysis. A sample was considered STEC-positive when it was positive for at least one of *stx1* or *stx2* genes. Data were pooled according to the sample type, and the level of significance set at 5%. All data were analysed with Stata 14 (StataCorp, College Station, TX, U.S.A.). Positivity rate of STEC contamination was estimated for

each sample type with exact binomial 95% confidence interval.

RESULTS

DUPLEX PCR SCREENING OF SELECTIVE ENRICHMENT BROTH CULTURES OF FIELD SAMPLES

The overall positivity rate of STEC contamination in selective enrichment broth samples originating from the abattoir and beef retail outlets was 35.2% (118/335; 95% CI: 30.1 - 40.6) and 12.5% (38/303; 95% CI: 9 - 16.8) respectively. The positivity rate of STEC by sample type ranged from 5.4% in dried cured beef broth samples to 40% in faecal broth samples (Table 1). The differences were statistically significant (p = 0.020) in beef retail outlets but not in abattoir samples (p = 0.240). Regardless of the sample-type, the most frequently detected genotype was *stx2* (30%; 102/335).

ISOLATION OF STEC STRAINS

Of the 118 and 38 *stx*-positive broth samples originating from abattoir and beef retail outlets, only 20% (24/118) and 21% (8/38) yielded 30 isolates for abattoir samples and 21 isolates for beef retail outlets, respectively. By sample type, carcass swab samples 29% (18/63) were more culturable compared with faecal samples 13% (6/46) for abattoir samples, and for retail samples dried cured beef samples were more culturable 40% (2/5), followed by ground beef 23% (5/22). Only 30 isolates were recovered from the abattoir sample types; 7 from faeces and 23 from carcass swabs but no isolate was recovered from the abattoir liquid waste. For the beef retail samples, 21 isolates were recovered from the following sample types: 6, 11 and 4 isolates from dried cured beef, ground beef and brisket flat cut, respectively (Table III). The dPCR results revealed that all the isolates harboured *stx* genes. The difficulty experienced in recovering STEC isolates from immune-concentrated magnetic bacteria beads suspension on SMAC, necessitated the use of CHROMagar. Despite the use of CHROMagar however, isolation was evidently low.

DISCUSSION

Various studies have demonstrated the dichotomy in PCR detection of STEC from different sample matrices (field and clinical samples) and the subsequent difficulty in recovering isolates from positive broth cultures (Fratamico *et al*., 2014; Li *et al*., 2017; Macori *et al*., 2020). Our results confirm this phenomenon and we attempt to summarize the different factors that may be associated with these discrepancies and consequently pose a risk to food safety and public health in general.

In the current study, we employed standard laboratory methods and treatments of field samples which included selective broth enrichment procedures that followed the Bacteriological Analytical Manual protocol (US-FDA, 2013) with slight modifications based on sample type. However, regardless of sample type, we observed only 20% (24/118) and 21% (8/38) culturable *stx*-positive broth cultures from the abattoir and beef retail outlets. Researchers have reported low recovery rate of STEC isolates in Europe (Ciupescu, Nicorescu, Mihai, Dumitrache and Tanasuica, 2015), the U.S.A. (Woo and Palavecino, 2013), Argentina (Brusa *et al*., 2017) and South Africa (Kalule, Keddy and Nicol, 2018). Our results were comparable with those of Hoang, *et al*., (2015) who reported 23.2% (13/56) *stx*-positive samples from raw beef and clinical samples in Japan. However, our results were lower than observations reported by Bosilevac and Koohmaraie, (2011) USA, who obtained 1,006 PCR *stx*-positive ground beef samples, with only 30% (300/1006) samples being culturable. Similarly, in China, Fan *et al*., (2019) reported 108 *stx*- positive PCR samples but only 53% (57/108) and 27% (29/108) by Chrom-STEC agar and MaConkey agar, respectively.

While it may not be possible to fully discuss the factors that contribute to PCR positive/culture negative discrepancies, this study have attempted to summarize few factors:

Firstly, a state of viable but non-culturable (VBNC) has been reported in bacteria. This occurs when certain bacteria specie enters a state of very low metabolic activity, due to unfavourable environmental conditions it is referred to as the viable but non-culturable (VBNC) state (Li *et al*., 2014; 2020). This is a survival mechanism employed by some pathogenic and non-pathogenic bacteria including STEC (Ramamurthy *et al*., 2014; Li *et al*., 2020) to enable long term survival in unconducive environmental conditions. In the VBNC state, there is loss of culturability, which undermines their detectability with routine agar thereby leading to underestimation of viable cell counts and recovery of isolates (Li *et al*., 2017b). Interestingly, VBNC has the capacity to revert to culturable state under favourable conditions (Ramamurthy *et al*., 2014; Li *et al*., 2020), and this represents a potential threat to food safety and public health systems.

The second factor is the presence of competing background microflora in food samples. Irrespective of the potential benefits of non-pathogenic population background microbial flora particularly in food sample, studies have shown that they could be some logarithmic cycles higher than the invading pathogenic bacteria (Pearson, 2013; Samelis *at al*., 2001). Consequently, the background microbial flora would predictable have a competitive advantage over the invading pathogenic bacteria for nutrient utilization, thereby modifying metabolic activities of the pathogens in the sample matrices, particularly meat samples or other foods (Joris *et al*., 2011, Nobilli *et al*., 2017). Accordingly, the cell would be stressed, occur in low numbers and oftentimes becomes undetectable even in media with increased

Table 1. Prevalence of Shiga toxin-producing *Escherichia coli* (STEC) by sample type collected from abattoirs and beef retail outlets in a one-year cross-sectional study in Gauteng Province, South Africa.

Sample type	Field broth samples	STEC (%)	95% CI	stx_1 (%)	stx_2 (%)
Slaughterhouse					
Faeces	115	46 (40)	30.9 - 49	33 (29)	39 (33)
Carcass swabs	201	63 (31.3)	24.8 - 37.8	38 (19)	55 (27)
Liquid waste	19	9 (47)	23 - 72	8 (42)	8 (42)
Total	335	118 (35.2)	30.1 - 40.6	79 (24)	102 (30)
Beef					
Dried cured beef	92	5 (5.4)	0.7 - 10	1 (1)	5 (5)
Ground beef	123	22 (17.8)	11 - 24.7	14 (11)	20 (16)
brisket flat cut	88	11 (12.5)	5.4 - 19.5	9 (10)	5 (6)
Total	303	38 (12.5)	9 - 16.8	24 (8)	30 (10)

(Feng *et al*., 2013; Dong *et al*., 2013). Genes for synthesis of the outer membrane O-antigens (wzx, wzy) can be used to discriminate STEC based on serogroup in molecular assays (Lin *et al*., 2011). Fast, sensitive, and specific PCR assays have been developed for such serogroup-based detection in food and carcass samples (Paton and Paton, 1998; Fratamico *et al*., 2011; Lin *et al*., 2011; Wang *et al*., 2013; Conrad *et al*., 2014).

The routine use of PCR as a diagnostic method is limited by cost and occasionally the accessibility of sufficient test sample volume. To reduce or eliminate these limitations a variant called multiplex PCR has been developed. In multiplex PCR two or more loci are simultaneously amplified in the same reaction, resulting in time saving. From 1998 when it was first developed (Chamberlain *et al*., 1998), this method has been effectively employed in many areas of DNA testing, including analysis of deletions (Chamberlain *et al*., 1990; Elnifro *et al*., 2000), mutations and polymorphisms (Shuber *et al*., 1993; Rithidech *et al*., 1997), as well as quantitative assays (Zimmermann *et al*., 1996; Sherlock *et al*., 1998). PCR has already also been used extensively for virotyping and pathotyping based on the detection of virulence genes hence the characterization of *E. coli*. It is therefore most suitable for studying complex pathogens such as *E. coli* with diverse strains and pathotypes.

Table 2: Sequences of primers used to detect virulence genes in Shiga toxin-producing *Escherichia coli* (STEC) strains

Primer	Sequence (5' - 3')	Size (bp)
stx_1F	ATA AAT CGC CAT TCG TTG ACT AC	180
stx_1R	AGA ACG CCC ACT GAG ATC ATC	
stx_2F	GGC ACT GTC TGA AAC TGC TCC	255
stx_2R	TCG CCA GTT ATC TGA CAT TCT G	

Table 3: Culturable stx-positive field broth samples, and toxin genes detected in isolates of Shiga toxin-producing Escherichia coli (STEC) recovered from various sample types

Sample type	No. of *stx*-positive broth samples	No. of Culturable samples (%)	No. of recovered Isolates	stx_1 (%)	stx_2 (%)
Slaughterhouse					
Faeces	46	6 (13)	7	4 (57)	5 (71)
Carcass swabs	63	18 (29)	23	19 (83)	12 (52)
Liquid waste	9	0	0	0	0
Total	118	24 (20)	30	23 (77)	17 (57)
Red meat					
Dried cured beef	5	2 (40)	6	2 (33)	6 (100)
Ground beef	22	5 (23)	11	2 (18)	11 (100)
brisket flat cut	11	1 (9)	4	4 (100)	0
Total	38	8 (21)	21	8 (38)	17 (81)

specificity and sensitivity, such as Chromogenic agar (Hirvonen *et al*., 2012; Gouali *et al*., 2013).

Furthermore, PCR assay is very sensitive. Food safety monitoring systems are very important; however, they are only as sensitive, dependable and feasible as their applied methodologies. The standard manuals established by the United States Department of Agriculture (USDA) recommend PCR and real-time PCR (qPCR) as screening methods for detection of STEC by targeting various genes

The ability of STEC to cause human disease is influenced primarily by the production of shiga like toxins *(stxs)*, which are encoded by *stx* genes carried on bacteriophages (or phage) (Melton-Celsa, 2014; Scheutz *et al*., 2012). Phages harbouring *stx* genes are defined as *stx* phages, and they are present in the intestinal microbiota of humans and other animals (Martinez-Castillo *et al*., 2013; Handley *et al*., 2012). Runoff water from the cattle farms contains large number of free *stx* bacteriophage. Phages which have been shown to be infective due to their capacity to generate lysogens and to transduce *stx* genes (Muniesa and Jofre, 1998; Imamovic *et al*., 2010; Martínez-Castillo and Muniesa, 2014). The high sensitivity of PCR assay, could detect free *stx*-carrying phages in meat samples (Pradel *et al*., 2000; Samadpour *et al*., 2006). It is also possible that the initial sample PCR is very sensitive and detects the presence of levels of STEC which are not detectable when testing a small number of colonies by PCR.

Finally, there is always a loss of *stx* genes after the first sub-culture step has been demonstrated (Bielaszewska *et al*., 2007; Joris *et al*., 2011) during the isolation from naturally contaminated bovine faecal samples. Other studies have also demonstrated that repeated sub-culturing or long-term storage could cause loss of *stx* gene (karch *et al*., 1992).) This may lead to an underestimation of STEC positive samples.

CONCLUSION

This study validates the PCR positive/ culture negative phenomenon, considering that even with the use of selective enrichment broth and Chromogenic agar which is reported to have increased specificity and sensitivity (Hirvonen *et al*., 2012; Gouali *et al*., 2013), recovery rate was still very low compared with the number of PCR positive samples. From an epidemiological perspective, it is recommended that the use of only PCR to detect STEC and virulence genes in broth cultures should be acceptable where isolation is not achievable. This is supported by other authors who opined that, this may be the best method for generating relevant epidemiologic data for disease control (Brusa *et al*., 2013, Martínez-Castillo and Muniesa, 2014).

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