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Original Article

Detection and Molecular Characterization of Sorbitol Negative Shiga Toxigenic *Escherichia Coli* in Chicken from Northwest of Iran

Mohammad Tabatabaei [1*]
Aram Mokarizadeh[2]
Nasim Foad-Marashi[3]

[1]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[2]PhD Candidate, Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
[3]Private Clinician, Urmia, Iran

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are food-borne pathogens primarily associated with the consumption of contaminated ground beef and are an important food safety concern worldwide. STEC has been found to produce a family of related cytotoxins known as Shiga toxins (Stxs). Shiga toxins have been classified into two major classes, Stx_1 and Stx_2. A single strains of STEC can produce Stx_1, Stx_2 (or its variants) or both. The aims of this project were to determine the prevalence and molecular characteristics of STEC isolates from chicken flocks in Northwest of Iran. A total of 350 fecal samples from 28 broiler farms were screened for the presence of STEC by conventional culture methods and polymerase chain reaction (PCR). All samples were initially subjected to phenotypical analysis using the Sorbitol MacConkey agar plate for the detection of the sorbitol negative *E. coli*, and then for genotypic analysis, by multiplex PCR for detection of *stx*1and *stx*2 genes. STEC were isolated from 14 (4 %) chicken fecal samples. To our knowledge, this is the first report of isolation of STEC from poultry in Iran. To conclude, this work revealed the presence STEC strains harboring *stx*1 and *stx*2 gene in healthy chicken fecal samples in Northwest of Iran suggesting they can play as an important potential source of contamination for people working on broiler farms or are in contact with chicken carcasses at meat processing plants.

Key words: *Escherichia coli*, Chicken, Shiga toxin-producing *E. coli*.

[*]Corresponding author:
Mohammad Tabatabaei , DVM, PhD
Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
E-mail address: mtabatabaei2003@yahoo.co.uk

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are food-borne pathogens primarily associated with the consumption of contaminated ground beef and are an important food safety concern worldwide. One of the common methods of transmission is consumption of contaminated beef and beef products.[1] STEC strains have been found to produce a family of related cytotoxins known as Shiga toxins (Stxs). Shiga toxins have been classified into two major classes, Stx_1 and Stx_2. An STEC strain can produce Stx_1, Stx_2 (or its variants) or both. The ability of STEC strains to cause serious disease in humans is related to the production of one or more Shiga-like toxins (Stx_1, Stx_2, or their variants), which inhibits protein synthesis of host cells, thus leading to cell death.[2]

STEC is currently considered as a major cause of food-borne disease, mostly in the United States, Canada, Japan and Europe.[3] Although most sporadic cases and outbreaks have been reported from developed countries, human infections associated with STEC strains have also been described in Latin American countries, including Argentina, Chile and Brazil.[3,4] It has also been reported from Kenya, Turkey and Iraq.[5-7]

Considering the clinical significance of the STEC, rapid, specific, and sensitive detection methods are required to identify toxin-producing isolates. The traditional approaches for detection of STEC strains are time consuming and laborious, requiring several days to weeks to identify the organism. In addition, the random selection for colourless colonies on SMAC plates may reduce the test sensitivity and unable to detect the phenotypic variants of *E. coli* O157:H7 which ferment sorbitol and produce pink colonies on SMAC agar plates.[8]

More recently, molecular approaches such as PCR-based methods, which detect *E. coli* O157:H7 based on the presence or absence of specific virulence genes such as the *stx* and *eaeA* genes, have been described.[9-11]

Presence of STEC strains in chicken fecal samples can play as an important potential source of contamination for workers of farms or chicken carcasses at meat processing plants. On the other hand, the limited information on the prevalence of STEC and *E. coli* O157:H7 in poultry products made an assessment of the risks difficult and the options for management and control unclear.

The presence of STEC strains in human fecal samples was first reported in 2005.[12] Isolation of *E. coli* O157 from dairy farms and ground beef was reported in Iran in 2007 and 2008, respectively.[13,14] Contamination of chicken carcasses with STEC strains have been reported from other countries,[15] but it has not yet been investigated in Iran. Therefore, in this study we investigated presence of STEC strains in chicken fecal samples in Northwest of Iran.

Materials and Methods

Sample collection. From May to September 2007, a number of 350 fresh fecal swab samples, from 28 broiler farms across the West Azerbaijan province, Northwest of Iran, were randomly collected and maintained in 0.9 % NaCl containing 10 % (v/v) glycerol and kept cold while being transported to the laboratory. The samples were processed on the same day, which collected.

Culture conditions. Immediately, each cloacal swab sample were aseptically transferred to 5 ml of trypticase soy broth containing 0.5 mg ml^{-1} novobiocin, followed by incubation at 37°C for 18 hrs. The enriched culture was plated onto MacConkey agar plate. After 18 hrs incubation at 37°C, from each plate 5 single pink colonies streaked onto SMAC agar plates supplemented with cefixime (0.05 mg ml^{-1}) and potassium tellurite (2.5 mg L^{-1}) (CT-SMAC). The inoculated CT-

SMAC plates were then incubated at 37°C for 24 hrs. Non sorbitol fermenter (NSF) colonies were selected from CT-SMAC plates and streaked onto eosin methylene blue (EMB) agar plates and were then incubated at 37°C for 24 hrs. These isolates, with typical *E. coli* metallic shine on EMB agar, were characterized by biochemical tests as described by Quinn et al.[16]

PCR assays. NSF colonies on CT-SMAC that had been confirmed as *E. coli* employed as templates for PCR assay. *E. coli* O157:H7 (ATCC-43895) (Bacterial collection of Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran) which contain *stx*1 and *stx*2 genes were used as the positive control and *E. coli* DH5α (Cinagen, Iran) as the negative control. A whole-cell suspension was prepared by suspending bacterial colony in 50 µl sterile distilled water. The cell lysate was made by heating the suspension for 10 min in a boiling water bath.[17] The lysate were spun for 10 min at 10,000 rpm to pellet the cellular debris. Two µl of the supernatant containing bacterial DNA was used as template for amplification by PCR. Oligonucleotide primers for *stx*1 and *stx*2 genes were synthesized as previously described.[11] Table 1 describes oligonucleotide sequence of primers used in the PCR reaction mixture.

Multiplex PCR (m-PCR) for detection of *stx*1 and *stx*2 genes was performed with a CP2-003 thermal cycler (Corbett Research, Australia). PCR assays were carried out as described by Kim et al.[18] with some modifications. Briefly, two microliters of nucleic acid templates prepared from new isolates and reference strains, 2.5 µl 10 × PCR buffer [10 mM Tris-HCl (pH 8.4), 10 mM KCl], 3 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM dNTPs, and 0.5 U of *Taq* DNA polymerase (Fermentas, Germany) were added in a 25 µl volume of reaction mixtures. PCR conditions consisted of an initial 95ºC denaturation step for 3 min followed by 35 cycles of 95ºC for 20 s, 58ºC for 40 s, and 72ºC for 90 s. The final extension was followed at 72ºC for 5 min. Amplified DNA fragments were resolved by gel electrophoresis using 1.5 % agarose gels in 0.5 × Tris Boric acid- EDTA (TBE) buffer. Gels were stained with 0.5 µg of ethidium bromide (EtBr) per ml, visualized and photographed under UV illumination. One hundred bp DNA ladder (Fermentas, Germany) was used as a size marker for PCR assay.

Specificity of PCR. To determine the specificity of the PCR assay, five different bacterial strains included *Pseudomonas* spp, *E. coli* DH5α, *Staphylococcus areous*, *Streptococcus* spp. and *Pasteurella multocida* were tested by PCR with same condition.

Results

Fourteen (4%) non-sorbitol fermenter STEC were isolated from 350 chicken fecal samples. All STEC isolates were examined for *stx*1 and *stx*2 genes by multiplex PCR (m-PCR). A number of 10 (2.8%) STEC isolates were positive for both *stx*1 and *stx*2 genes. Three (1.16%) of the STEC isolates were positive for *stx*1 gene, and *stx*2 gene was only detected in one isolate (0.28%). Results of the m-PCR are shown in Fig. 1 and Fig. 2; the m-PCR assay generated two PCR products with a length of 614 bp and 779 bp, indicating the presence of *stx*1 and *stx*2 genes of STEC strains, respectively. The specificity of PCR primers targeting *stx*1 and *stx*2 gene was determined by testing five different bacterial strains. The m-PCR amplified the expected 614 bp and 779 bp fragment from the reference *E. coli* strain, while none from the other bacterial strains was amplified.

Table 1. Primers used in multiplex PCR for *stx*1 and *stx*2 genes

Primer	Oligonucleotide sequences (5'-3')	Expected size	Reference
*stx*1-F	ACACTGGATGATCTCAGTGG	614 bp	11
*stx*1-R	CTGAATCCCCCTCCATTATG		
*stx*2-F	CCATGACAACGGACAGCAGTT	779 bp	11
*stx*2-R	CCTGTCAACTGAGCAGCACTTTG		

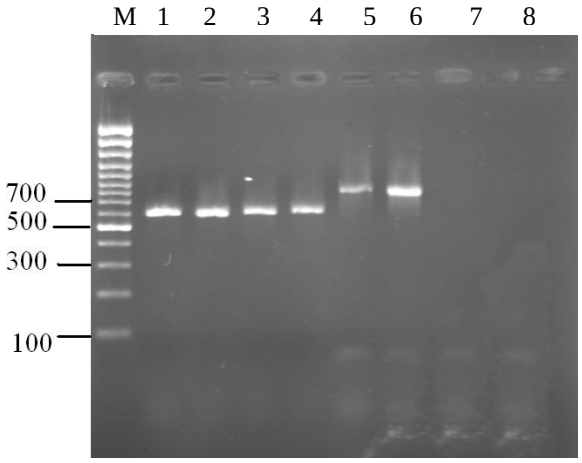
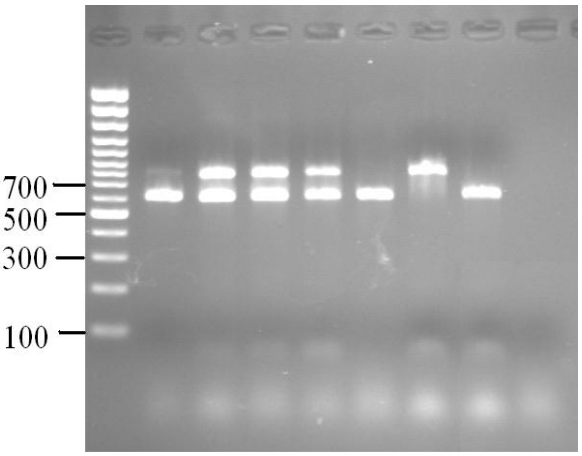


Fig 1. PCR analysis of *E. coli* strains isolated from chicken fecal samples. Lane M: 100bp DNA ladder, Lanes: 1- 3 ETEC isolates with *stx*1 gene, lane 4 positive control with *stx*1 gene (614 bp), Lane: 5 ETEC isolate with *stx*2 gene, Lanes 6 positive control with *stx*2 gene (779 bp), lane 7 and 8 negative control with stx1 and stx2 primers, lane 9 negative control without DNA template.

M 1 2 3 4 5 6 7 8

Fig 2. Multiplex PCR analysis of *E. coli* strains isolated from chicken fecal samples. Lane M:



100bp DNA ladder, Lanes: 1, 5 and 7, ETEC isolates with *stx*1 gene (614 bp), Lane: 6 ETEC isolate with *stx*2 gene (779 bp), Lanes 2, 3 and 4 ETEC isolates with *stx*1 and *stx*2 genes, lane 8 negative control without DNA template.

Discussion

In poultry production facilities the occurrence of *Salmonella* spp.[19] and *E. coli* O157:H7[20] in faecal samples is well documented, but there is not information about prevalence of STEC in chicken faeces in Iran. This study has found that the prevalence of STEC was not as high as that of other countries. However, Shiga-toxin producing *E. coli* has been isolated from 4 % of poultry fecal samples. Our results suggested that chicken, like many other countries, could be a reservoir of STEC strains in Iran, [21-23], but little research has been performed on the quantification of this pathogen in the chicken faeces worldwide. Although direct extracts of faeces or foods can be used as templates for PCR, the best results are usually obtained by testing extracts of primary broth cultures.[24,25] Broth enrichment serves two purposes: inhibitors in the sample are diluted, and bacterial growth increases the number of copies of the target sequence. It has been proposed that the enrichment before plating on selective agar may increase the sensitivity of *E. coli* O157:H7 isolation compared to direct plating of test samples on selective agar. It has been described that the CTSMAC agar medium yields the best results for selective cultivation of *E. coli* O157:H7.[26,14]

PCR is generally considered to be the most sensitive means of determining whether a faecal specimen or a food sample contains STEC. To date, different PCR assays have been described for detecting the main virulence factors gene such as *stx*1 and *stx*2 genes encoding accessory STEC virulence factors, such as *eaeA*, *hlyA* and *flic*H7. [18, 27] Previous studies have shown that PCR assays based

on *stx* gene sequences can detect the presence of very low numbers of STEC organisms in microbiologically complex samples.[10,29] According to these information we recognized fourteen STEC strains with *stx*1, *stx*2 *or stx*1 and *stx*2 genes by a multiplex PCR. Using this PCR assays for identification of STEC that have been employed in this study, might be a possible alternative to immunological assays for detection of STEC. Further studies are needed to clarify this possibility. The STEC strains seem to be pathogenic for human only if they possess accessory virulence factors, thus there is a need more study to evaluate STEC isolates for *eae*A, *hly*A and *flic*H7 genes.

To conclude, this work revealed the presence of STEC strains harboring *stx*1 and *stx*2 gene in healthy chicken fecal samples in Northwest of Iran confirming they can play as an important potential source of contamination for people working on farms or are in contact with chicken carcasses at meat processing plants. Therefore, more careful investigation programs should be applied to check all chicken meat processing plants and markets. Continuous monitoring and surveillance program for examining microbial contamination of imported feeds should be performed to minimize the risk of spread of major food borne pathogens.

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