# PCR analysis of mutton and chicken samples for the presence of Shiga Toxigenic *E. coli*

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## Abstract

Shigatoxigenic *Escherichia coli* strains are major food-borne pathogens that are transmitted through contaminated meat and meat products. Apart from cattle, sheep and goat are important natural reservoirs. Faecal contamination during slaughter and further processing along with poor hygienic practices accounts for the presence of STEC in raw meat. A total of 200 samples (50 each of mutton and chicken and 50 samples each of mutton and chicken swabs) collected from various sources were subjected to PCR analysis for the presence of Shigatoxigenic *E. coli* using primers specific to STEC virulent genes, shiga toxin (*stx*1 and *stx*2) and enterohaemolysin (*hly*A) with amplification size of 614bp, 779bp and 361bp respectively. Out of 200 samples, 58 showed presence of STEC (20 mutton, 24 mutton swabs, 7 chicken and 7 chicken swabs out of 50 samples each). Of the 58 STEC positive samples, 42 (72.4%) showed presence of *stx*1, 16 (27.5%) showed *stx*2, 21 (36.2%) showed *hly*A gene and 8 (13.7%) showed both *stx*1 and *stx*2. Among 58 STEC positive isolates, 8.6% (1 mutton, 2 mutton swab and 2 chicken swabs) isolates were possessing all the 3 virulent genes. The sensitivity of PCR method for STEC was 1.7cfu. Enrichment with mTSB broth containing novobiocin gave good results compared to mEC broth with novobiocin. It is advisable to use more rapid, sensitive and specific PCR method to detect virulent STEC from different food sources.

**Key words:** STEC, PCR, mutton, chicken, shiga toxin, mTSB and mEC.

## Introduction

Strains of shiga-toxingenic *E. coli* (STEC) of different serotypes are recognized as important human pathogens of animal origin and can cause severe diseases such as Haemmorhagic Colitis, Haemolytic Uraemic Syndrome and Thrombotic Thrombocytopaenic Purpura [5]. *E. coli* O157:H7 is an important serotype of STEC which accounts for 70-80% of recognized clinical diseases [6]. It has been reported that >200 serotypes of *E. coli* including O157 and non-O157 STEC produce shiga toxins *stx*1 and *stx*2 [12] which are also known as verotoxins.

*E. coli* strains carrying *stx*2 gene along with enterohaemolysin (*hly*A) gene are potentially dangerous to human health. Shiga toxins bind to receptors on the bowel mucosa and are elaborated and translocated into the cell interior and inactivate ribosomal RNA leading to the inhibition of protein synthesis in cells expressing gly*coli*pid $G_3b$ (globotriaosylceramide) and eventually causes death of host cells [11, 14, 18]. High levels of $G_3b$ are found in human kidney which is 1000 times more sensitive to the cytotoxic action of *stx*2 than that of *stx*1 [10].

Blood released due to mucosal damage is lysed by enterohaemolysin liberating heme and haemoglobin which facilitates rapid multiplication of the organism [1, 13]. As multiplication takes place, further toxin production occurs, causing greater damage, releasing increasing amounts of blood and resulting in growth stimulation [9].

Epidemiological studies have shown that food of animal origin is the main source of human infection [7]. The organisms can enter human food chain from a number of intestinal contents before, during or after slaughter. That is why food of animal origin must be regarded as potential vehicle of STEC.

The present investigation was carried out for the presence of STEC in mutton and chicken samples collected from in and around Hyderabad, Andhra Pradesh.

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## Materials and Methods

A total of 100 meat samples (50 samples each of mutton and chicken) and 100 meat surface swabs (50 each of mutton and chicken swabs) were collected from freshly dressed and washed animal carcasses at slaughter houses and markets in Hyderabad. Meat samples (10gm each) and swabs were enriched in 90 ml of modified *E. coli* (mEC) broth and modified Tryptic Soy broth (mTSB) both supplemented with novobiocin at 37ºC for 18 hours. An *E. coli* O157:H7 strain, obtained from National Institute of Enteric diseases, Kolkata was used as known positive strain in PCR analysis. All the enriched samples were subjected to PCR analysis for the presence of STEC by using oligonucleotide primers against three virulence genes i.e. *stx*1, *stx*2 and *hly*A (**Table.1**) with an amplification size of 614, 779 and 361bp respectively as shown in **fig-1 & 2**.

**TABLE.1.** Oligonucleotide primers used in the study.

| Primer | Target gene | Primer sequence (5`-3`) | Fragment size (bp) | Reference |
|---|---|---|---|---|
| O157-3 | *hly*A | GTA GGG AAG CGA ACA GAG | 361 | Wang et. al., 1997 |
| O157-4 | *hly*A | AAG CTC CGT GTG CCT GAA | 361 | Wang et. al., 1997 |
| Stx1-F | *stx*1 | ACA CTG GAT GAT CTC AGT GG | 614 | Manna et. al., 2006 |
| Stx1-R | *stx*1 | CTG AAT CCC CCT CCA TTA TG | 614 | Manna et. al., 2006 |
| Stx2-F | *stx*2 | CCA TGA CAA CGG ACA GCA GTT | 779 | Manna et. al., 2006 |
| Stx2-R | *stx*2 | CCT GTC AAC TGA GCA CTT TG | 779 | Manna et. al., 2006 |

1.5 ml of enriched broths were taken into eppendorf tubes and bacteria were

were pelleted by centrifuging at 6000rpm for 5 min. and supernatant was discarded. To the pellet 50µl of molecular grade water was added and kept in a water bath at 65ºC for 15 min. and snap chilled. Then centrifuge at 13000rpm for 5 min. and the supernatants were used as DNA templates for PCR analysis.

Bacterial DNA amplification was done in 20µl reaction mixture containing 2µl of 10X Taq DNA polymerase buffer (containing 100mM Tris with pH 9.0, 500mM KCl, 15mM MgCl$_2$ and 1% Triton X-100), 2µl of 10mM of dNTP mix, 0.9U of Taq DNA polymerase (Genei), 2µl each of 4 p.moles/µl of forward and reverse primers and 5µl of crude bacterial cell lysate. Make this mixture to 20µl using molecular grade water. Amplification was done in thermal cycler following standardized conditions (**Table.2**).
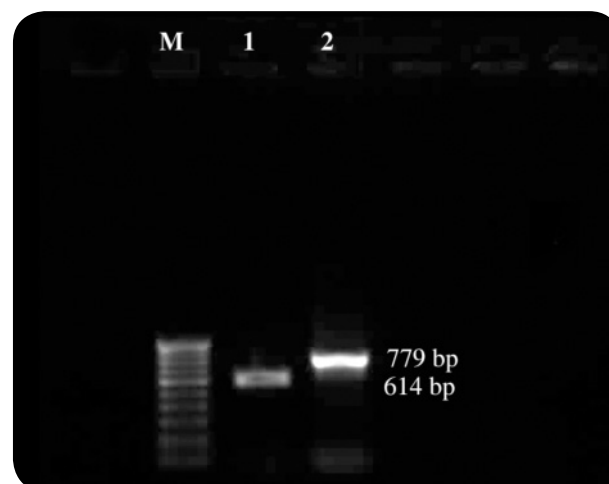


**FIGURE 1.** Results of the PCR assay showing stx1 and *stx2* bands

Lane M: 100-bp DNA ladder
Lane 1: amplifying 614-bp segment of *stx* 1
Lane 2: amplifying 779-bp segment of *stx* 2
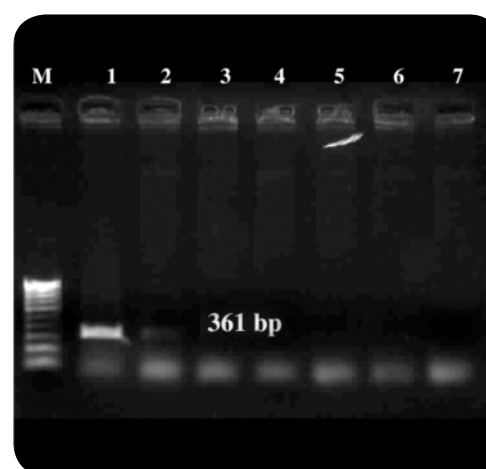


**FIGURE 2.** Results of the PCR assay showing *hly*A band.

**TABLE.2.** Cycling conditions used for three sets of primers.

| S.No. | Step | *hly* A | *stx*1 and *stx*2 |
|---|---|---|---|
| 1. | Initial denaturation | 940C/5min | 940C/5min |
| 2. | Final denaturation | 940C/1min | 940C/1min |
| 3. | Annealing | 520C/1min | 600C/1min |
| 4. | Initial extension | 740C/2min | 720C/2min |
| 5. | Final extension | 740C/10min | 720C/10min |

The amplified DNA fragments were resolved by agarose (1%) gel electrophoresis, stained with ethidium bromide (0.5μg/ml) and visualized with an UV transilluminator (**fig.1-4**).

**Sensitivity and Spiking studies:** To know the sensitivity of PCR assay for detection of STEC, homogenized beef was inoculated with known *E. coli* O157:H7 strain in 10gm of mutton and transferred to two different enrichment media, mEC and mTSB broths. The PCR analysis was carried out after 10hr and 18hr of incubation period.

## Results

The minimum detection level of STEC by PCR was 17cfu and 1.7cfu for mEC broth after 10hr and 18hr incubation respectively, where as it was 1.7cfu for mTSB after 10hr as well as 18hr incubation period. The results for sensitivity studies were depicted in fig-4. The growth of STEC in samples as well as spiking studies was good in mTSB compared to mEC as shown in **fig-3.**

The results for the presence of STEC in different meat samples are presented in **Table.3**. In this study, STEC were detected in 40% (20 out of 50) and 48% (24 out of 50) of mutton samples and mutton swabs respectively. Also STEC were detected in 14% (7 out of 50) of both chicken and chicken swab samples. Of the total 29% (58 out of 200) STEC positive samples (meat and meat swabs together), 72.4% (42 out of 58 STEC) samples showed presence of *stx*1, 27.5% of STEC (16 out of 58) showed *stx*2 and 13.7% of STEC (8 out of 58) showed presence of both *stx*1 and *stx*2.

**TABLE.3.** Occurrence of STEC virulent genes in different meat samples.

| S. No. | Sample | No. of samples | STEC | *stx*1 | *stx*2 | *hly*A | Both *stx*1& *stx*2 | All the 3 genes |
|--------|--------|----------------|------|--------|--------|--------|---------------------|-----------------|
| 1. | Mutton | 50 | 20 | 15 | 5 | 2 | 2 | 1 |
| 2. | Mutton swabs | 50 | 24 | 18 | 6 | 12 | 3 | 2 |
| 3. | Chicken | 50 | 7 | 5 | 2 | 2 | 1 | 0 |
| 4. | Chicken swabs | 50 | 7 | 4 | 3 | 5 | 2 | 2 |
| | Total | 200 | 58 (29) | 42 (21) | 16 (8) | 21 (10.5) | 8 (4) | 5 (2.5) |

The numbers in parenthesis represent percent values

When tested for presence of '*hly*A' by PCR, 36.2% (21 out of 58) of the total STEC positive samples showed presence of *hly*A. The *hly*A gene was detected in 10% (2 out of 20) of STEC positive mutton isolates, 50% (12 out of 24) mutton swab isolates, 28.5% (2

Lane M: 100-bp DNA ladder
Lane 1 : Positive control (*E. coli* O157:H7)
Lane 2: amplifying 361-bp segment of *hly*A
Lane 3-7: negative controls

614 bp

**FIGURE 3.** Evaluation of PCR compatibility of enrichment broths for detection of STEC (*stx*1).

Lane 1: 100-bp DNA ladder
Lane 2 &3: amplifying 614-bp segment of *stx* 1 enriched in mEC broth
Lane 4 & 5: amplifying 614-bp segment of *stx* 1 enriched in mTSB broth
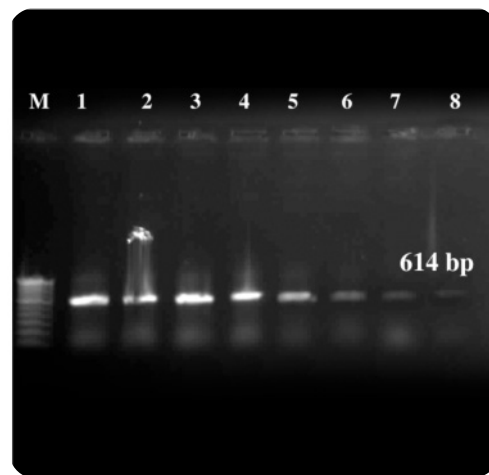
614 bp

**FIGURE 4.** Evaluation of threshold sensitivity of PCR assay (*stx*1).

out of 7) chicken isolates and 71.4% (5 out of 7) chicken swab isolates. Among 58 STEC positive isolates, 8.6% (1 mutton, 2 mutton swab and 2 chicken swabs) isolates were possessing all the 3 virulent genes.

## Discussion

STEC are the cause of acute infections in humans such as HC, HUS and thrombotic thrombocytopenic purpura [15]. They can also induce subclinical infections and mild diarrhea [7]. Foods of animal origin are the most common vehicle of STEC infection in humans. It has been isolated from wide variety of foods such as beef, mutton, chicken, pork, eggs, milk and milk products. Detection of STEC from food stuffs is problematic, since they are present at low level together with large number of competing microflora and also they may be injured by different food processing methods. Traditional methods for detecting and identifying STEC are labour intensive and time consuming. Hence there is a need to develop reliable and rapid methods for detection of STEC from foods.

Commercially available rapid antigen-antibody test methods such as ELISA and lateral flow immuno precipitation and molecular methods like PCR have reduced the time and labour involved in the analysis of food products. These assays still require enrichment of the food samples but significantly reduce the overall assay time compared with conventional methods [3]. In contrast to culture based methods, PCR methods may also detect cells that are non-culturable [4]. The polymerase chain reaction is a sensitive, rapid technique in which a few copies of target DNA can be amplified to a level detectable by gel electrophoresis [8]. The PCR represents a major advance in terms of the speed, sensitivity and specificity of diagnostic methods and has been increasingly used to identify several bacterial species from food and clinical samples [17].

In the present study, the sensitivity of PCR was found to be 1.7cfu after 18hr incubation period in both mEC and mTSB broths. The sensitivity and specificity of STEC isolates by PCR was better with mTSB compared to mEC broth. In this study, mutton samples yielded high percentage of STEC compared to chicken samples. Many studies have shown that sheep are major reservoir of STEC [16]. The $stx$1gene was found more frequently in this study compared to $stx$2. Most of the published test studies also revealed the higher production of $stx$1 than $stx$2 [2, 16].

Since the *E. coli* strains carrying the shiga toxin genes along with enterohaemolysin (*hly*A) gene are potentially dangerous to human health, PCR is a very specific and rapid method of detecting the virulent STEC. The detection of STEC in foods of animal origin in this study warrants that human diarrhoeal stools during foodborne outbreaks must be routinely examined for STEC.

## Conclusion

This study emphasizes the prevalence of virulent STEC in mutton and chicken samples in and around Hyderabad, India. STEC are present in many raw foods at low levels and frequencies and keeping in view the very low infectious dose of *E. coli* O157 (< 10 viable bacteria), much greater efforts are required for detection of these STEC. The possible ways of entry of various *E. coli* serotypes could be handling of meat and meat products by adopting improper hygienic measures during handling and processing. Therefore, careful processing of meat and meat products is an important public health measure. Proper hygienic measures need to be adopted for handling various food products in order to bring down the entry of these organisms in to meat, thereby safeguarding the human beings from health hazards.

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