Research Article

Prevalence of Shiga Toxin Producing Enterohemorrhagic *Escherichia coli* O157:H7 Isolated from Chicken Meat in Northern Punjab, Pakistan

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Abstract | Food borne diseases related to *Escherichia coli* pursue to be one of the most significant global level public health issue in the world. Shiga toxin producing *Escherichia coli* (STEC) produces a principal virulence factor shiga toxin (*Stx*), which can lead to diarrhea, haemolytic uremic syndrome (HUS), hemorrhagic colitis (HC) and other lethal complications. Consumption of STEC contaminated food has been associated with food related illnesses outbreak. This study aims to evaluate the prevalence of O157:H7 strain of STEC by detection of *Stx-1* and *Stx-2* genes in 160 chicken meat samples, which are randomly collected from different regions of Northern Punjab, Pakistan. Isolation of pathogen from meat samples were performed with the use of International Organization for standardization based microbiological techniques, while chain reaction technique (PCR) was used for detection and characterization of *Stx-1* and *Stx-2* genes. In total, 75 (46.8%) isolates were detected as *E. coli*. Among them, 14 (8.75%) isolates were tested positive for *Stx* genes (*Stx-1* and *Stx-2*). The detection of pathotype of shiga toxin producing *E. coli* O157:H7 in chicken meat is a significant finding because this pathogen has been related to food borne outbreaks. On the basis of our findings, routine diagnosis of STEC and improvement of hygienic measures must be considered as a critical concern for public health.

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Keywords | Chicken meat, Diarrhea, *E. coli*, STEC O157:H7, *Stx-1* and *Stx-2* genes

Introduction

Escherichia coli (*E. coli*) an opportunistic pathogen has a symbiotic relation with animals and human intestinal tract. The extra-intestinal and diarrhoeagenic (enteric) infections instigated by this pathogen are aggregating (Denamur *et al*., 2021). The categorization of diarrhoeagenic *E. coli* into diverse pathotypes are founded on pathogenicity mechanism and virulence traits. Along with, other constituted are Shiga toxin-producing *E. coli* (STEC), its subgroup enterohemorrhagic *E. coli* (EHEC), enteroaggregative

E. coli (EAEC) and enteropathogenic *E. coli* (EPEC) (Haiwen *et al*., 2019; Koutsoumanis *et al*., 2020).

STEC are described through the generation of Shiga toxins (*Stx*) (Jinnerot *et al*., 2020). These are food borne pathogen which causes many diseases in humans (Da Silva *et al*., 2022; Ramatla *et al*., 2022). Shiga toxins are grouped into two types, *Stx*1 and *Stx*2, subsidized to diverse degrees of virulence, which are further assembled into several subtypes e.g. *Stx*1a to *Stx*1d and *Stx*2a to *Stx*2k (Ori *et al*., 2019; Yang *et al*., 2020). In humans, mostly the STEC infections symptoms are bloody or severe diarrhea, stomach ache, hemorrhagic colitis (HC), end stage renal disease (ESRD) and nevertheless probably life-threatening complications known as haemolytic uremic syndrome (HUS) developed by 6–25% of patients, delineated thru thrombocytopenia, bloody diarrhea, acute kidney injury and haemolytic anemia (Lu *et al*., 2022; Yin *et al*., 2014; Zhang *et al*., 2021). In foodborne outbreaks in the European Union (EU), in humans STEC was the third most perceived bacterial agent with 7775 cases reported (The European Union One Health 2019 Zoonoses Report, 2021).

E. coli O157:H7 is an antecedent STEC serotype and has the capability to harbor antibiotic resistance and virulence genes and can cause haemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) worldwide (Bolukaoto *et al*., 2019; Perera *et al*., 2015). Diarrhea in association with HUS has been considered as most important cause of acute renal failure in healthy children in the United States (Monet-Didailler *et al*., 2019). STEC infection once has developed and is followed by HUS are unpreventable and diarrhea that is associated with HUS cannot be treated (Monet-Didailler *et al*., 2019).

STEC strain infection transmission occurs commonly through direct/indirect contact, intake of food products specially through improperly cooked meat, vegetables, game meat, milk, dairy products (Elafify *et al*., 2022) and contaminated water with feces of carriers (Dias *et al*., 2022). The *Stx*2 is more harmer, and a mixture of both toxins in specific ratio is usually associated with Hemolytic uremic syndrome (Andreoli, 2022). STEC is responsible for 90% of pediatric HUS because of additional presence of GB3 receptors as compared to adults and old (Lu *et al*., 2022).

For causing cytotoxicity, STEC toxins make them capable of causing death through the blockage of cell's ability of protein synthesis (Shen *et al*., 2022). In January 1993, Outbreak related to STEC O157:H7 was appeared for the very first time due to consumption of improperly cooked hamburger. In 1999, masses of habitats in New York were found STEC O157:H7 positive due to drinking of water which was polluted with cattle manure (Abd El-Moez, 2022).

Considering the pathogenic role of *E. coli* in causing severe lethal diseases. The aim of this study is the investigation of prevalence of shiga toxin producing *E. coli* (STEC) O157:H7 strain in chicken samples from the different areas of Northern Punjab, Pakistan.

Materials and Methods

Sample collection and transportation

In this cross-sectional study, a total of 160 raw meat samples of chicken were randomly collected from abattoirs, various butchers and supermarkets of different areas of Northern Punjab, Pakistan. The collection and transportation of samples were according to recommendations of 'Pakistan standard and quality control authority' (PSQCA) (PS/CAC/GL 50-2004 General guidelines on sampling). Approximately, 50g of chicken meat was taken into sterile containers. All samples were brought in cooler at 4°C. In the laboratory the samples were processed promptly.

Sample preparation and microbial analysis

25g of chicken samples was added to a sterile conical flask containing 10 ml of Tryptone-soy broth (Sigma Aldrich, USA) and were kept in the incubator at 37°C for 18-24 h. After that, 500µl volume was taken from that which was poured and spread on to the prepared MacConkey agar (Sigma Aldrich, USA) culture plates and incubated for 24-36 h in the incubator at 37°C. Lactose fermenting bacteria formed red to pink color colonies bordered by acid precipitated bile zones on the MacConkey agar plate. These colonies were picked and streaked on to prepared Sorbitol MacConkey (SMAC) (Sigma Aldrich, USA) agar culture plate, incubated for 24h at 37°C. All culture media used were autoclaved at 121°C. The sorbitol fermenting organisms grown into pink colonies while sorbitol non-fermenting organisms e.g. *E. coli* O157:H7 grown colorless. *E. coli* colonies were analyzed morphologically and microscopically using Microscope (IREMCO IM910, Germany).

DNA extraction

The colorless colonies on sorbitol MacConkey agar were picked and put in to LB broth (Sigma Aldrich, USA) and incubated for 24hr in the incubator at 37°C to get the turbid solution having heavy growth of bacteria. For the extraction of DNA, pellet was obtained from the turbid solution by centrifugation in the centrifuge machine (HERMLE Z216MK, Germany) for 5 min at 10,000rpm. It is followed by cell lysis using 450µl of 1X TE buffer at 8 PH (10mM Tris HCl and 0.5M EDTA) and 50µl of 10% SDS. The mixture was incubated for 1h at 37°C. Protein was removed by using 500µl of phenol-chloroform solution in 1:1 ratio. After doing vortex through vortex machine (IREMCO, Germany) for few seconds the suspension was centrifuged for 20 min at 10,000 rpm. Upper aqueous phase was used to precipitate DNA by using 300µl ice cold isopropanol and 50µl of 3M sodium acetate. It is followed by centrifugation for 5 min at 13000 rpm. After careful removal of upper liquid phase, tubes were washed with 1 ml 70% chilled ethanol and centrifugation was done at 10,000 rpm for 1 min. Ethanol was removed and tubes were kept for 30 min for drying. DNA pellets were re-suspended in 100 to 200µl of TE buffer containing RNase. DNA pellets were stored at -20°C.

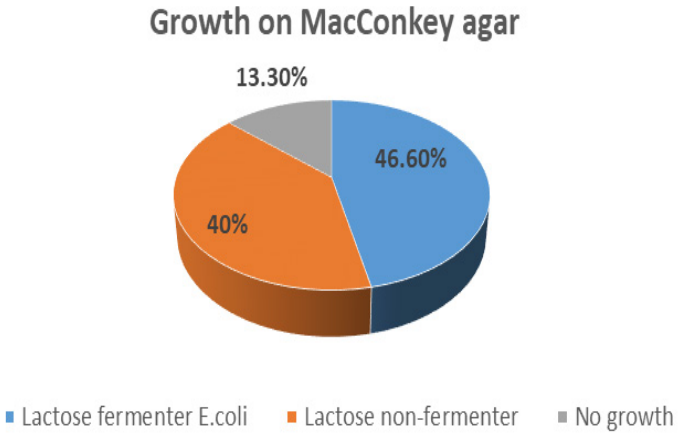

Figure 1: *Pie-chart showing the growth on MacConkey agar.*

Molecular detection through PCR

For detecting pathotypes of *E. coli*, two virulence genes *Stx1* and *Stx2* were detected using primers through polymerase chain reaction (PCR) (Tahamtan *et al.*, 2010) using Thermocycler (Multigene optimax Labnet international, Inc. USA). The detection of virulence genes *Stx1* and *Stx2* were done with a master mix of total volume 25µl by using PCR. 25µl of master mix contain 1 µl from each forward and reverse primers, 5 µl of PCR buffer, 0.5 µl of Taq polymerase, 1 µl dNTPs, 2 µl of template DNA and 14.5 µl of double distilled water. For PCR optimization, initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing of primers at 54°C for 30s, extension at 72°C for 30s and final extension at 72°C for 10 min. The amplification product of PCR was electrophoresed in 1.5% agarose gel with staining of ethidium bromide using Gel Doc (InGenius3, Cambridge, UK), and then visualized under UV light.

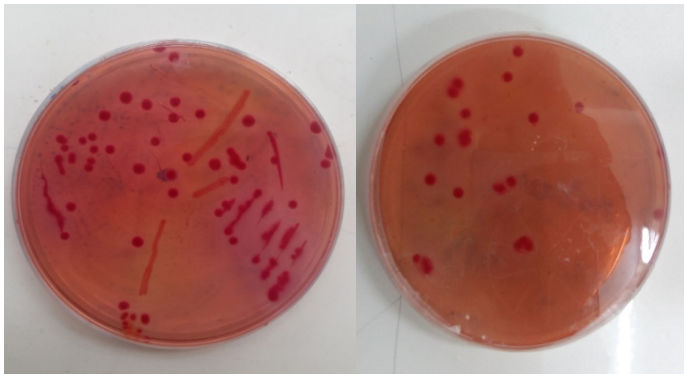

Figure 2: *Growth of lactose fermenter E. coli on MacConkey agar.*

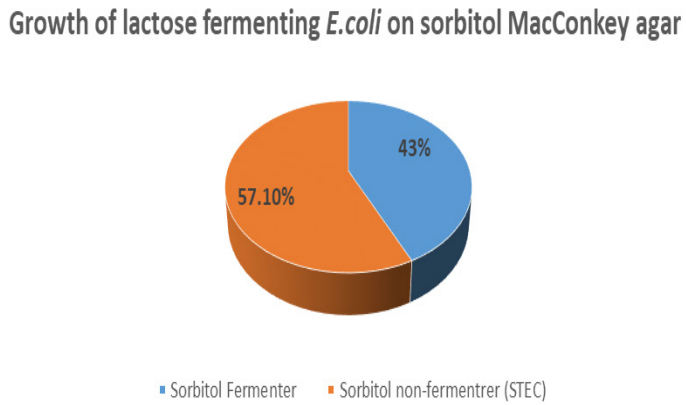

Figure 3: *Pie-chart showing growth of lactose fermenting E. coli on sorbitol MacConkey agar.*

Table 1: *Description of primers used for Stx1 and Stx2 genes (Tahamtan et al., 2010).*

Name of primers		Sequence	Size
Stx-1	*Stx*1 F	5'-CTTCGGTATCCTATTCCCGG-3'	484 bp
	*Stx*1 R	5'-GGATGCATCTCTGGTCATTG-3'	
Stx-2	*Stx*2 F	5'-CCATGACAACGGACAGCAGTT-3'	779 bp
	*Stx*2 R	5'-CCTGTCAACTGAGCAGCACTTTG-3'	

Results and Discussion

Out of 160 raw meat samples 75 (46.8%) isolates were identified as *E. coli*. On the basis of serological and microbial identification, 35 (46.6%) isolates were identified as lactose fermenter. All lactose fermenters grown into red or pink colonies with acid precipitation bile zones (Table 1, Figure 1 and 2).

20 (57.1%) isolates among these lactose fermenters were sorbitol non-fermenter (STEC). While 15 (43%) isolates were sorbitol fermenters (Figure 3). All of these STEC isolates have formed colorless colonies on sorbitol MacConkey agar (Figure 4).

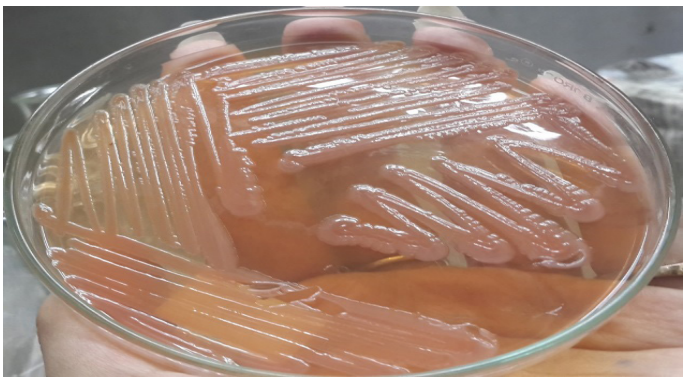

Figure 4: *Colorless colonies of STEC on sorbitol MacConkey agar.*

Prevalence and characterization of Stx genes

Among these sorbitol non-fermenters, 7 (9.33%) samples were found to have *Stx-1* (Figure 5) and 7 (9.33%) samples were found to have *Stx-2* (Figure 6) genes. These *Stx-1* and *Stx-2* genes presence in sorbitol non-fermenting *E. coli* confirmed the STEC pathotype *E. coli* O157:H7.

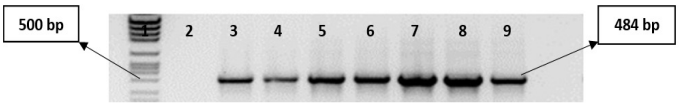

Figure 5: *Confirmation of Stx-1 (484bp) in 7 samples through PCR (1= marker, 2= control, 3 to 9=chicken samples).*

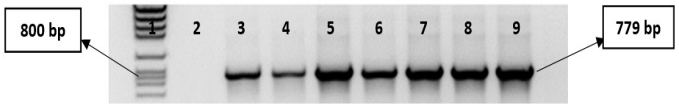

Figure 6: *Confirmation of Stx-2 (779bp) in 7 samples through PCR (1=marker, 2=control, 3 to 9 chicken samples).*

Overall prevalence of E. coli O157:H7 in raw chicken

Among all the 160 chicken samples processed, 14 (8.75%) samples were found to have *Stx* genes (*Stx-1* and *Stx-2*). While 146 (91.25%) samples were found negative for having *Stx* genes.

In the current study, 8.75% of chicken meat samples were detected to have shiga toxin *E. coli* O157:H7 isolates. This detection in chicken meat is a significant finding because this pathogen has been related to food borne outbreaks. Diarrheagenic *E. coli* based food borne outbreaks exhibit an important public health problem. The importance of this O157:H7 pathotype of *E. coli* is exacerbated at their low infective dose, severity level of clinical manifestation and case mortality rate (Madoroba *et al.*, 2022).

In the current study, although the prevalence of this *E. coli* isolate is not remarkable, this infection rate is significant from public health perspective. The prevalence of this STEC isolate in present study agreed with the studies in Iran, Korea and some other countries (Zarei *et al.*, 2021; Lee *et al.*, 2009). According to their studies, the prevalence of STEC in poultry sample is 5.3% and 7.3%, respectively. However, the results of current study are different from studies held in Iran and other countries (Momtaz and Jamshidi, 2013). Their study reported that 21% of samples have STEC isolates among 422 samples, their study also found that 96% of isolates has *Stx-1* gene (Momtaz and Jamshidi, 2013). In contrast, in our current study, only 8.75% samples have *Stx-1* gene. One of the causes for this frequency contrast can be the differences in the total number of specimen studied. However, it is reported by Guran *et al.* (2017) that the prevalence of STEC O157:H7 was 1.3% (Guran *et al.*, 2017).

In the present study, the strains *E. coli* O157:H7 were found positive for *Stx-1* and *Stx-2* genes. In India, it is reported by Dutta *et al.* (2011) that 33.3% isolates have atleast one virulence gene. 23.8% of these isolates were found as STEC (Dutta *et al.*, 2011). Similarly, in South Africa, Madoroba *et al.* reported that 0.5% samples of raw and processed meat were tested positive for *E. coli* O157:H7 (STEC) (Madoroba *et al.*, 2022).

It can be the reflection of poor hygienic practices during various phases at abattoir from slaughtering, handling procedures, shipment, processing and preparation of meat (Galarce *et al.*, 2021). Although *E. coli* is present in the gastrointestinal tract of humans and animals and is nonpathogenic inhabitant, consumption of food or drinking water contaminated with pathogenic *E. coli* such as STEC can cause lethal gastrointestinal diseases, such as haemolytic uremic syndrome (HUS),

hemorrhagic colitis (HC) and diarrhea (Elsharawy *et al*., 2022). STEC is thought to be one of the most common pathogenic microbe which is transmitted through poultry meat to humans.

Continuous optimization, improvements and advancement of methods which are culture based for STEC isolation are recommended. It is suggested as this may be significant for risk-based research and population structure understanding, extent of disease potential, characteristics, severity and pathogenicity of these pathogens.

Conclusions and Recommendations

In summary, this research highlighted the presence of STEC strain O157:H7 in raw chicken meat. This is significant from the standpoint of One Health because of the meat contamination multiplex nature along the whole value chain. This is extremely necessary to control STEC as it is very threatening for chicken consumers. It is recommended to improve the hygienic measures during different stages of meat processing and Development of model which is efficient and can be utilized for STEC routine diagnosis to check the contamination of meat.

Novel technologies and Policy management for detecting STEC can be the focus for future research for better understanding of population-based epidemiology of pathogen.

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Novelty Statement

Prevalence of shiga toxin producing *E.coli* in chicken meat have not been previously reported for northern Punjab, Pakistan.

Author's Contribution

Balquees Kanwal wrote the main article, Syeda Saba formatted the manuscript. And Syed Waqas and Farzana Shaheen has proofread the manuscript.

Ethical approval

The ethical approval is not necessary for conducting this study.

Conflict of interest

The authors have declared no conflict of interest.

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