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Research Article

MOLECULAR DIAGNOSTICS OF FOODBORNE PATHOGENS

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Abstract

All over the world Shiga toxin producing *E. coli* (STEC) are frequently cause of GIT illnesses in human originating in food of animals mainly from poultry.The aim of this study was to render rapid diagnostics of Shiga toxin producing *E. coli* (STEC) from raw meat and milk. During a 4 month period from May to August a total of 300 samples were collected from beef (100), chicken (100) and milk (100) and analyzed for Shiga toxin producing *E. coli*. Of the total of 300 samples of raw meat and milk only chicken (2%), beef (1%) and milk (1%) were detected as PCR positive.Raw meat and milk could be a source of Shiga toxin producing *E. coli* which indicates that possible risks of infections to people could be transferred by the consumption of raw meat and milk and their rapid diagnostics could be made possible by the use of rapid diagnostic technique polymerase chain reaction.

Introduction

In the intestinal micro flora of humans and mammals *Escherichia coli* (*E. coli*) are predominantly found.

A German pediatrician 'Theodor Escherich' in 1885 discovered *E. coli* belonging to a family Enterobacteriaceae and this bacterium is one of the inhabitants of intestine. These commensals never cause a problem in their host but some pathogenic *E. coli* are involved in diarrhea and other enteric illnesses and are called diarrheagenic *E. coli.* It harmlessly colonizes the intestine but several strains of *E. coli* cause various intestinal diseases. *E. coli* are described as Gram negative, facultative anaerobe, non spore forming; rod shaped and are generally motile. [1]. It has been divided into six pathotypes that includes atypical enteropathogenic *E. coli* (ATEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic or Shiga toxin producing *E. coli* (EHEC/STEC) and enteroinvasive *E. coli* (EIEC) [2]. These pathotypes indicate the plasticity of *E. coli* genome and the genome of STEC O157:H7 contains 5416 genes in $5.5x10^6$ base pairs of DNA [3] which is considered the most prominent and notorious STEC and its incidence varies according to age group and in most of the cases the etiological agent is food stuff [4].

Shiga toxin producing *E. coli* (STEC) is responsible for causing an increasing number of human outbreaks, characterized by bloody diarrhea, non-bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) and 85% of these cases are implicated with food borne transmission. *stx* is further subdivided into two families' stx1 and stx2 on the basis of sequence analysis. The cardinal virulent factors, of STEC are *stx1* and *stx2* and are referred as cytotoxic [2, 5].

The association of STEC outbreaks has been considered with a variety of dairy products especially

raw meat and milk if they are not properly cooked or boiled [6]. A number of reports have been submitted on the sources of transmitting the STEC such as water [7], yogurt [8] and airborne [9] as well. Human outbreaks of STEC are more derived from ground beef than any other source [6]. In particular, ruminants and cattle are considered the primary source of *E. coli* O157:H7 [10, 11]. It was also reported that person to person contact is also a source for the transformation of STEC and it was also manifested that STEC is shed by the infected person through stool even after a month of recovery [12].

Due to number of constraints, the conventional approaches for detection of foodborne pathogens were replaced by polymerase chain reaction in advanced countries. In Pakistan, however, the PCR has not been currently evaluated for its efficacy for the detection of foodborne pathogens from beef, mutton, chicken and milk due to the paucity of information on the PCR based detection of foodborne pathogens. The conventional approaches to detect food related bacteria rely on the selective enrichment and culture characteristics followed by biochemical characterization. These methods are time consuming, labor intensive and often not reliable in contrast to PCR which is a rapid molecular test with high sensitivity and specificity.

Material and method

A total of 300 samples including raw chicken (100), beef (100) and milk (100) were collected in PBS from various regions of Lahore and were carried to the laboratory under refrigerated conditions. After the enrichment of these samples in tryptic soya broth, were streaked on MacConkey agar for the identification of *E. coli* and then on Sorbitol MacConkey agar for the differentiation of pathogenic and non pathogenic *E. coli* after overnight incubation at 37°C aerobically. The confirmation of *E. coli* was done by conducting two biochemical tests, the catalase test using a drop of 3% hydrogen peroxide and gently mixed with the colonies of *E. coli* isolated of plates and a triple sugar iron test by preparing a butt to the $3/4^{th}$ portion of the test tube and then gently pulled back the loop and streaked on the slant area without lifting up the loop. The tubes were plugged properly with cotton plug and incubated at 37°C for overnight aerobically.

Finally, the detection of STEC (*Stx1* and *Stx2*) was confirmed by employing a molecular approach polymerase Chain Reaction which proved to be a rapid and reliable method for the detection of foodborne bacterial pathogens. For the detection of Shiga toxin1 (*stx1*) and Shiga toxin2 (*stx2*) on all the isolates of *E. coli* from the samples of raw meat and milk, a molecular approach was conducted i.e. polymerase chain reaction (PCR). This molecular approach was designed for the detection of virulence genes of *E. coli* isolates. Primers with unique sequences were used for the detection of the targeted genes and a primer sequence is available in tabular form in (Table#1). All PCR reagents were supplied by Fermentas USA and all PCR assays were performed in PCR Master Cycler (Eppendorfs Germany).

KS7 and KS8 primers were used for the identification of *stx1* gene. Final mixture of PCR reaction was 25 µl with 2.0 µl bacterial cells as template, 15.3 µl autoclaved water, 2.5 µl of 10X *Taq* buffer, MgCl2 1.5 µl, 0.5 µl of dNTPs (5 mM each of dATP, dCTP, dGTP, dTTP), 1.5 µl each of forward and reverse primer (20 pmol) and 0.2 µl of DNA *Taq* polymerase (5.0 U/ µl). Thermal cycler conditions used for *Stx1* were, Initial denaturation was adjusted at 95°C for 10 min. *stx1* gene was amplified for 30 cycles, each

cycle with 30 seconds of denaturation at 94°C, 1 min for primer annealing at 52°C and 1min for extension at 72°C followed by final extension at 72°C for 5 min and for Stx2 using primers LP43 and LP44, After initial denaturation at 95°C for 10 minutes amplification of DNA product for each cycle were for repeated 30 cycles with denaturation at 94°C for 30 seconds, primer annealing at 57°C for 1 minute, extension at 72°C for 1.5 minutes followed by final extension at 72°C for 5 minutes.

Results

Of the total 300 samples of raw chicken, beef and milk cultured on MacConkey agar showed 78, 65 and 57 lactose fermenting pink colonies of *E. coli* respectively. These colonies were then incorporated for further confirmation of *E. coli* using biochemical tests.

Triple sugar iron test (TSI) was performed on these pink colonies of *E. coli* for the confirmation of *E. coli* in which yellow color of the medium in all test tubes was observed which indicated the fermentation of all three sugars such as sucrose, lactose and glucose that leaded to the determination of *E. coli*. In addition to TSI, another biochemical test, catalase test was conducted on the same number of pink colonies for the confirmation of *E. coli* and a moderate reaction was observed with precipitation or bubbling which indicated the presence of *E. coli.*

After these biochemical confirmations these rose pink colonies of *E .coli* were further preceded for the determination of STEC using molecular methods. All plates with pink colonies of chicken (78%), beef (65%) and milk (57%) were streaked on SMAC. SMAC displayed Sorbitol fermenting (SF) rose pink colonies 53, 33 and 28 respectively and non-Sorbitol fermenting (NSF) colorless colonies 25, 32 and 29 respectively. These NSF colonies of chicken, beef and milk were detected as PCR positive with 2% of chicken, 1% of beef and 1% of milk, harboring STEC genes either *stx1* or *stx2* while none of the SF colonies were detected as PCR positive.

Discussion

Foodborne infections are caused by the consumption of contaminated food with bacteria, parasites and viruses (15). Shiga toxin is a deadly bacterial strain usually causes foodborne illnesses and often associated with beef contamination, giving weekend state to the young, weak or elders. *E. coli* appears in various forms like O157, producing Shiga toxin along with the emergence of HUS (16).

The pathogenicity of *E. coli* O157:H7 is ascribed with some virulence factors such as Shiga toxins Stx 1 and Stx 2 (17) which are encoded with Stx family and cytotoxic effects are manifested by this pathotype on endothelial cells of kidney, heart and brain (2). From 300 samples of raw meat and milk *E. coli* was identified using plate methods incorporating MacConkey agar, Sorbitol MacConkey agar, triple sugar iron test and catalase test. Sorbitol MacConkey agar differentiated NSF and SF. These NSF colonies were further tested for PCR as these colonies were regarded as pathogenic.

Table 1: PCR primers used in this study

Target gene	Primer name	Sequence 5-3	Amplicon size	References
Stx1	**KS7 (F)** **KS8 (R)**	CCCGGATCCATGAAAAAAACATTATTAATAGC CCCGAATTCAGCTATTCTGAGTCAACG	**282bp**	[13]
Stx2	**LP43(F)** **LP44(R)**	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	**584bp**	[14]

Isolation frequency of *E. coli* (STEC) from various sources

Sampling source	sample collected	+ve for *E. coli* on MAC	+ve for *E. coli* on TSI	+ve for *E. coli* Catalse	+ve for *E. coli* SMAC	PCR +ve	percentage
Chicken	100	78	78	78	53SF/25NSF	0/2	2%
Beef	100	65	65	65	33SF/32NSF	0/1	1%
Milk	100	57	57	57	28SF/29NSF	0/1	1%
Total	300	200	200	200	114/86	o/4	4%

It is revealed from a study that from 220 samples of diarrheagenic patient only 59% was having *E. coli* using MacConkey agar as initial identification. Sorbitol fermenting and non-sorbitol fermenting phenotype was identified on SMAC (18).

In present study the collection of samples was done between the months June to August and it was noted the positive testing was prevalent during these months. The elevation in ambient temperature is more favorable and plausible for incidence of STEC, thereby resulting in the constant source of infections. According to this information on elevation of temperature, the prevalence of STEC could be expected more, as observed, in summer season from June to August and this pathogen STEC was found in this duration to the detectable level in raw meat and milk.

In a survey of United Kingdom, it was reported that the positive testing of *E. coli* O157:H7 was only in the months between May and September. The positive testing of *E. coli* O157:H7 was more prevalent between June-September in United State (19).

In present study for the identification of STEC a PCR approach was employed using gene specific primers targeting virulent genes *Stx1* and *Stx2* and monitored with a reference strain EDL933. The specificity of PCR was validated using a reference strain and was further evaluated on 300 isolates of raw meat and milk of different geographical regions. The PCR approach was conducted for rapid and reliable identification of STEC on enrichment of samples instead of using DNA isolation procedures and plate labor intensive methods to replace the traditional tedious techniques. Out of 300 samples of chicken, beef and milk only 4 (1.3%) number of samples were detected as positive harboring STEC genes either *stx1* or *stx2*. Of the 4 (1.3%), hundred samples of each

chicken, beef and milk carried STEC 2%, 1% and 1% respectively.

Detection methods based on nucleic acid have been developed for nearly all bacterial pathogens, based on hybridization assays PCRs targeting the gene region or specific sequence of a gene. Several problems may be encountered for the direct detection of pathogens from the food samples: contamination in sensitive PCR assay, PCR inhibition by food components and detection of living as well as dead cells (20). As described above two virulent genes were selected from the bacterium Shiga toxin producing *E. coli*: *Stx1* and *Stx2*. By incorporating the PCR primers and other components, became able to develop a PCR for the detection of these genes. An important factor to evaluate DNA based test is the specificity of DNA sequence chosen for gene of interest which is going to be targeted (Winters *et al*., 1998). The ability of PCR is to detect or amplify a gene or a segment of a gene directly from the sample containing various bacterial strains. One major problem in detecting the gene directly from the sample which often encountered with PCR is the reduction in sensitivity (21).

To overcome the PCR inhibition problems and to increase its sensitivity, enrichment method was employed. Enrichment method increased the number of required viable count while dead organisms reduces the probability of detection and even 2µl of the enrichment was able to produce the PCR results which indicate the PCR sensitivity and specificity.

In conclusion, a rapid, simple and convenient PCR-based method was developed for the specific detection of one of the major food borne pathogens. This method rendered final results in hours rather than lengthy and equally expensive biochemical methods. Application of this method in food

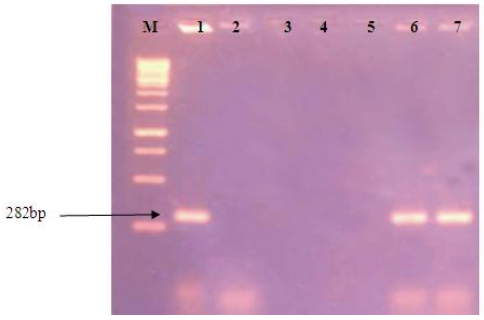

Fig.1: PCR (Chicken). **Lane M:** Molecular weight marker of 1kb. (Fermentas-GeneRuler, 0.1 µg / µl, 50 µg), **Lane 1:** Amplification product of 282 bp for *Stx1* gene in positive control strain EDL933, **Lane 2:** No amplification in negative (Master Mix-Template) control strain, **Lane 6 & 7:** Showing amplification product of 282 bp of *Stx1* gene, **Lane 3,4 & 5:** Showing empty wells

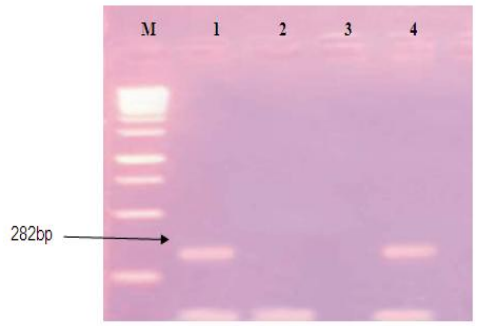

Fig. 2: PCR (Beef).**Lane M:** Molecular weight marker of 1kb. (Fermentas-GeneRuler, 0.1 µg / µl, 50 µg), **Lane 1:** Amplification product of 282 bp for *Stx1* gene in positive control strain EDL933, **Lane 2:** No amplification in negative (Master Mix-Template) control strain, **Lane 3:** Showing empty well, **Lane 4:** Showing amplification product of 282 bp of *Stx1* gene.

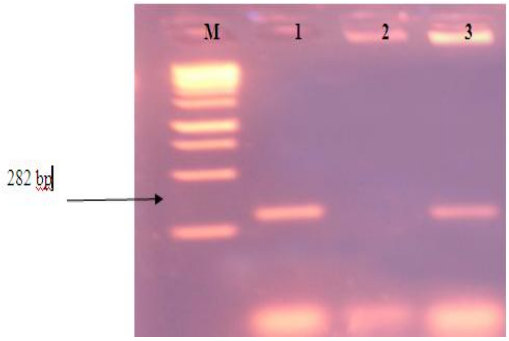

Fig. 3: PCR (Milk) **Lane M:** Molecular weight marker of 1kb.(Fermentas-GeneRuler, 0.1 µg / µl, 50 µg), **Lane 1:** Amplification product of 282 bp for *Stx1* gene in positive control strain EDL933, **Lane 2:** No amplification in negative control strain (Master Mix-Template), **Lane 3:** Showing amplification product of 282 bp of *Stx1* gene.

industries and municipal water supply departments is an additional benefit attributed to this techniqu

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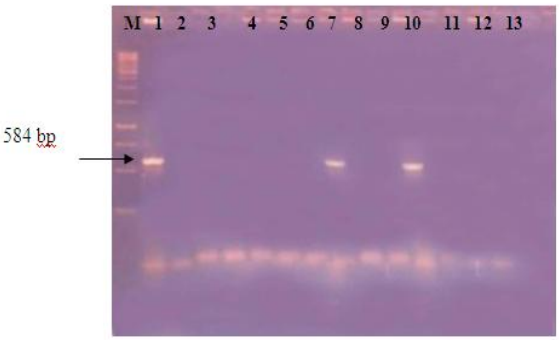
Fig. 4: PCR (Chicken) **Lane M:** Molecular weight marker of 1kb.



(FermentasGeneRuler, 0.1 µg / µl, 50 µg), **Lane 1:** Amplification product of 584 bp for *Stx2* gene in positive control strain EDL933, **Lane 2:** No amplification in negative control strain (Master Mix-Template), **Lane 7 &10:** Showing amplification product of 584 bp of *Stx2* gene, **Lane 3,4,5,6,8,9,11,12 & 13:** Showing negative samples.

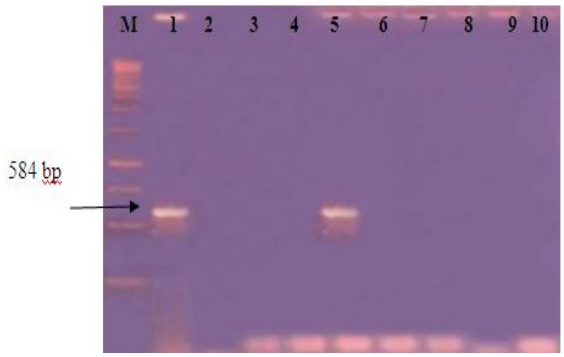


Fig.5: PCR (Beef). **Lane M:** Molecular weight marker of 1kb. (Fermentas-GeneRuler, 0.1 µg / µl, 50 µg), **Lane 1:** Amplification product of 584 bp for *Stx2* gene in positive control strain EDL933, **Lane 2:** No amplification in negative control strain (Master Mix-Template), **Lane 5:** Showing amplification product of 584 bp of *Stx2* gene, **Lane 3,4,6,7,8 9& 10:** Showing negative samples.

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