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ISOLATION AND MOLECULAR CHARACTERIZATION OF SHIGA TOXIN PRODUCING *E. COLI O157:H7* IN RAW MILK USING mPCR

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ABSTRACT: Raw milk may contain pathogenic microorganisms, probably harmful to humans inclusive of *Escherichia coli O157:H7* inflicting hemorrhagic colitis and hemolytic-uremic syndrome. Raw milk is believed to be a high risk food as it is highly nutritious and results in growth and multiplication of different microorganisms. *E. coli O157:H7* infect humans by different mode of transmission. Present study was performed to estimate the prevalence and the molecular characterization of *E. coli O157:H7* retrieved from retail raw milk samples in Quetta Pakistan. Out of 100 raw milk samples, 12 (12%) possess positive colorless colonies on CT-SMAC. Biochemical tests for the confirmation of *E. coli O157:H7* alongside gram's staining of the colonies had been accomplished. The amplification of isolates by mPCR using universal specific primers, *stx1* and *stx2* confirmed that 6 (50%), 3 (25%) and 3(25%) were positive for *stx1*, *stx2* and both *stx1* and *stx2* genes respectively. It is concluded that raw milk harbor *E. coli O157:H7*, having shiga toxin genes.

INTRODUCTION: Milk being the solitary remnant of human food used among all ages of life and plays an important role in the Pakistani diet [1]. Around 50% of the milk manufacture is devoured as fresh or boiled, 1/6th as yoghurt or curd and persisting is used to manufacture in different kinds of milk products, like khoa, paneer, butter, kheer, ice cream, gulabjaman, rabri and burfi. The products are prepared through conventional methods without hygienic quality of products; allow growth and multiplication of different microorganism [1, 2].

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Raw milk is consumed among farm families as they believe raw milk and their products are healthier over pasteurized one [3]. Due to the presence of nutrients and water, milk act as culture medium for growth and multiplication of different microorganism [2]. Microorganisms may enter raw milk through damaged udder, or by different stages be it from the animal, milkier (human as well as automated), external dirt or unhygienic processing of milk and water [3].

Among number different microorganisms *Escherichia coli* (*E. coli*) are the most frequently contaminating organism on normal flora of intestinal tract of warm blooded animals [4-5]. *Escherichia coli O157:H7* become first identified in 1982 as a human pathogen [6], since then considered a major concern of dairy and food industries due to its capability of causing serious

illness particularly hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Many outbreaks associated to EHEC *O157:H7* are related to consumption of undercooked meat, as cattle are reservoir of *O157:H7* [7-9], raw milk and their produces, water, alfalfa sprouts, lettuce and apple juice [10]. *E. coli O157:H7* are pathogenic due to production of several virulence factors, together with shiga toxin genes (*stx1* and *stx2*) or Verocytotoxins (VT1 and VT2), simulating a main role in pathogenesis of HC and HUS along cytotoxic effect on kidney cells, as well as central nervous system, intestines and other organs cells [9] along attaching and effacing lesions [11].

As a result of escalate in investigation of *E. coli O157:H7* cases, considerable notice has been stated to establish approaches for detection of this pathogenic bacterium, including cultural isolation, DNA probes, serological tests and polymerase chain reaction (PCR) assays. PCR based approach are rapid, sensitive, specific and automated method for identification of microbial pathogens [12-13]. The use of milk and their products is locally increasing routinely. In view of the developing public knowledge regarding food quality and safety, awareness on milk microbial and chemical composition is of serious importance for additional evolving of its hygienic organizing into excellence consumer products. Yet, information on such manner is limited and scattered. Hence, the objectives of present study are to:

Investigate the prevalence of pathogenic bacteria *E. coli O157:H7* in raw milk of Quetta Pakistan.

Molecular characterization of *E. coli O157:H7* using pair of specific, *stx1* and *stx2* primers.

MATERIAL AND METHODS:

Collection and Preparation of Samples: Total of 100 retail raw milk samples were collected from June to august in sterile glass bottles aseptically from different areas in Quetta Pakistan. All the collected samples were processed within 24 hr in the laboratory adopting all possible hygienic measures during collection, transportation and processing of samples. The study was carried out at "Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB)" University of Balochistan, Quetta.

Culturing, isolation and biochemical characterization/identification: For *E. coli O157:H7* detection, 25 ml from each milk sample was homogenized in 225 ml of modified tryptone soy broth (mTSB; Oxide, England) supplemented with 0.5 mg/ml novobiocin and incubated at 37 °C for 24 hr. After 24 hr, the broth was observed for turbidity and growth. A loopful from mTSB broth was streaked on sorbitol MacConkey agar (SMAC; Oxide, England) supplemented with 0.05 mg l^{-1} cefixime and 1.25 mg l^{-1} potassium tellurite (CT-SMAC) [14] and incubated at 37 °C for 24 hr. Two to three typical sorbitol negative colorless colonies [15] were sub-cultured on CT-SMAC agar plates for purification of serotype *O157:H7* isolates. Finally, colorless colonies were streaked on EMB (eosin methylene blue; Oxide, England) agar plates and incubated for 24 hr at 37 °C. The typical metallic shine *E. coli* on EMB agar plates were stained with Gram's stain and confirmed by several biochemical tests as shown in **Table 5** [16].

DNA Extraction: DNA was extracted using standardized cetyltrimethyl ammonium bromide (CTAB) method as described by Ausubel *et al.*, (2003) [17]. Briefly, 1 ml of nutrient broth inoculated and saturated with biochemically confirmed *E. coliO157:H7* isolates was transferred to eppendorf tubes (1.5 ml) and centrifuged for 2 min. The obtained supernatant was discarded and the pellet was resuspended in 500 µl 1 x TE buffer, 70µl of 10% SDS, 50µl of (20 mg/ml) proteinaseK and incubated at 60 °C for 1 hr. 100 µl of 5 M NaCl and 100 µl of 10 % CTAB/NaCl solution were added, mixed well, and incubated for 10 min at 65°C. An equal quantity (0.8 ml) of Phenol/chloroform/isoamyl alcohol (25:24:1) was added to extract DNA and Centrifuged for 10 min at 12000 rpm.

The aqueous upper phase was transferred to new tubes and equal quantity (0.7 ml) of cold isopropanol was added and incubated at -20 °C for 30 min to precipitate the DNA. The precipitate was pelleted by spinning at 15,000 rpm at 4 °C for 10 min. The DNA pellet was washed with ethanol (70%) after discarding supernatant and respun at

15,000 rpm for 10 min to re pellet the DNA. The pellet was air dried overnight after discarding supernatant. Finally in 100 µl 1 x TE buffer pellet was resuspended and stored at –20 °C until used for amplification.

Multiplex Polymerase Chain Reaction (mPCR): The extracted DNA form *E. coli O157:H7* was used for amplification of stx producing *stx1* and *stx2* genes using two specific pairs of oligonucleotide primers [18] synthesized and supplied by Macrogen, Korea. The sequence of oligonucleotide primers and size of amplified PCR products are listed in **Table 1**. Multiplex-PCR amplification was carried out in 25 µl volume of reaction mixture containing the contents listed in **Table 2**.The mPCR thermal cycling was performed using an automated 2720 thermal cycler (Applied Bio system, Singapore). For each mPCR run, PCR grade water was used in reaction mixture instead of DNA template as negative control and positive control, culture of locally isolated *E. coli O157:H7* producing both *stx1* and *stx2* toxins was obtained from CASVAB, university of Balochistan, Quetta. The reaction mixture was subjected to 35 cycles for the amplification of *stx1* and *stx2* genes using the following appropriate conditions listed in **Table 3** [19].

TABLE 1: OLIGONUCLEOTIDE - PRIMERS SEQUENCE USED IN PRESENT STUDY FOR AMPLIFICATION OF FRAGMENTS OF *STX1* AND *STX2* TOXIN PRODUCING GENES AND THE PREDITED SIZE OF AMPLICONS PRODUCED

Primer	Primer sequence (5'-3')	Amplicon size	Reference
stx1 F	CAG TTA ATG TGG TGG CGA AG	513-bp	33
stx1 R	CTG TCA CAG TAA CAA ACC GT		
stx2 F	TTC TTC GGT ATC CTA TTC CC	482-bp	33

TABLE 2: COMPOSITION OF 25µL REACTION MIXTURE FOR PCR. TOTAL VOLUME OF 25µL REACTION MIXTURE WAS PREPARED BY USING THE FOLLOWING REAGENTS

Reagent	Quantity	Concentration
PCR H_2O (Molecular grade)	13.75µl	--------
PCR Buffer (10X)	2.5µl	1X
$MgCl_2$ (25 mM)	2.5µl	2.5mM
dNTPs (10mM)	2µl	50 µM each
Stx1 F primer (20 pmol/µl)	0.5µl	0.4 pmol
Stx1 R primer (20pmol/µl)	0.5ul	0.4 pmol
Stx2 F primer (20 pmol/µl)	0.5µl	0.4 pmol
Stx2 R primer (20pmol/µl)	0.5ul	0.4 pmol
Taq DNA polymerase (5U/µl)	0.25µl	1.25U(0.05U/ul)
DNA Template	2µl	-------
Total volume	25µl	-------

TABLE: 3 MULTIPLEX-PCR CYCLING CONDITIONS FOR THE AMPLIFICATION OF *STX1* AND *STX2*

Conditions	Temperature & time
Initial Denaturation	95 °C for 5 min
Denaturation	94 °C for 45 sec
Annealing	50 °C for 45 sec
Extension	72 °C for 45 sec
Final Extension	72 °C for 7 min

TABLE 4: CHARACTERIZATION OF ISOLATED *E. coli O157:H7* BY mPCR FROM RETAIL RAW MILK SAMPLES

Number of collected raw milk samples	*E. coli O157:H7*	PCR of *E. coli O157:H7*		
		stx1	stx2	stx1 and stx2
100	12(12%)	6(50%)	3(25%)	3(25%)

TABLE 5: BIOCHEMICAL CHARACTERIZATION OF *E. COLI O157:H7*

Biochemical test	Reaction
Citrate Utilization	-ve
o-nitrophenyl-Dgalactopyranoside(ONPG)	+ve
Indole production	+ve
Voges-Proskauer (VP),	-ve
Methyl red (MR)	+ve
Nitrate reduction	+ve
Urease	-ve
Ornithine decarboxylase	+ve
Lysine decarboxylase	+ve
Arginine	+ve
Dulcitol	-ve
Catalase	+ve
Oxidase	-ve
Sorbitol	-ve
Rhamnose	-ve
Manitol	+ve
Glucose	+ve
Arabinose	+ve
Gelatin	-ve
Triple sugar iron (TSI)	-ve

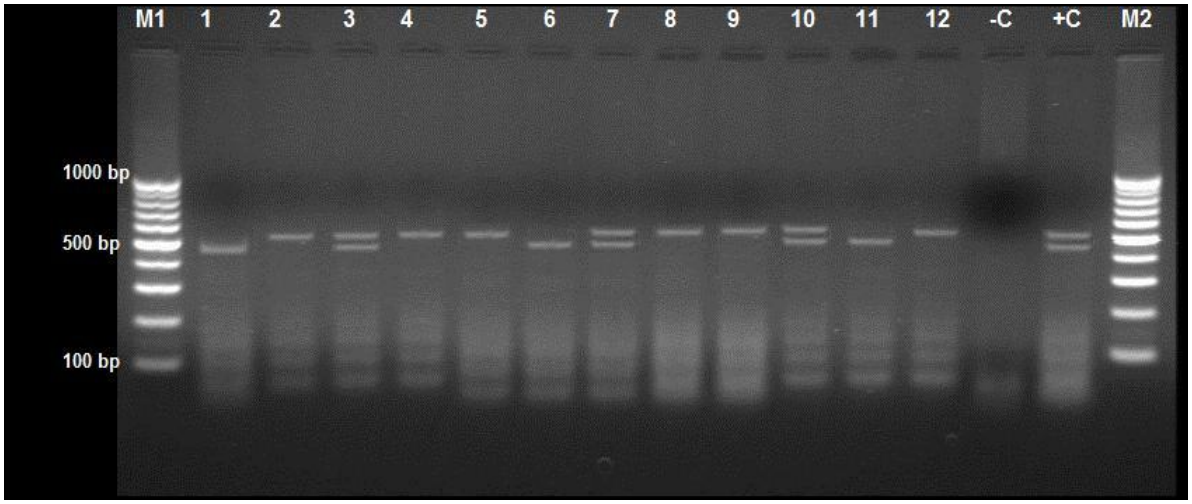

FIG. 1: CONVENTIONAL PCR: AGAROSE GEL (2%) ELECTROPHORESIS SHOWS AMPLIFICATION FRAGMENTS OF STX1 (513 bp) AND STX2 (482 bp) GENES BY DUPLEX PCR. LANES 2, 4, 5, 8, 9 & 12 POSITIVE AMPLIFICATION OF STX1. LANES 1, 6 & 11 POSITIVE AMPLIFICATION OF STX2. LANES 3, 7 & 10 POSITIVE AMPLIFICATION OF BOTH STX1 AND STX2. M1/M2: 100 bp DNA MARKER. +C: POSITIVE CONTROL. –C: NEGATIVE CONTROL

Gel Electrophoresis: The amplified PCR products mixed with 6 x tracking dye (Novagen, USA) were loaded and separated along with 100-bp DNA marker (vivantis, Malaysia) at 100 V for 1 hr by electrophoresis on 2% (W/V) agarose gel involving ethidium bromide 0.5 µg/µL in 1 x TAE buffer. The electophoresed amplicons were visualized and documented on UV transilluminator (Weal tec Dolphin-View, USA) [13].

RESULTS AND DISCUSSION: Total of 100 raw milk samples collected from retail shops of Quetta, Pakistan and tested for prevalence of *E. coli O157:H7*. The mPCR results showed that prevalence of *E. coli O157:H7* was 12 (12%) in retail raw milk samples as shown in **Table 4** and **Fig. 1**.

Conventional culture method using primary enrichment on mTSB showed 12 (12%) sorbitol non fermenting white colorless colonies on plates of CT-SMAC agar and green metallic sheen on EMB agar. Each of the isolate was gram negative and rod shaped [20]. The isolated bacteria gave

different reaction in biochemical Tests listed in **Table 5**. Several samples of raw milk contaminated with *E. coli O157:H7* showed different areas of city Quetta is experiencing unhygienic milk from retail shops. Different factors cause milk contamination like pitiful hygienic milking conditions, milking utensils, contaminated equipments, milk handlers hands' personal poor hygiene [21-22]. The samples collection was carried out during summer months, which had been related with an apex in the number of cows, protecting STEC [23]. Therefore, contamination of milk with *E. coli* 0157:H7 could be much less frequent at other occasions of the 12 months. Our findings do not differ greatly from those reported in other countries. It was reported by Padhye & Doyle 1991, that prevalence of *E. coli O157:H7* was 11/115 (10%) from raw milk samples, signifying a prevalence level akin to our data [24].

In another study Abid *et al.,* 2009 examined prevalence of *O157:H7* 14 (8.75%) from 160 milk samples produced by farmers from different commercially available brands in city of Peshawar, agreement to present study [25]. Studies conducted on raw cow, goat and buffalo milk reported contamination of 6.21% (11) out of 177 milk samples with serotype *O157:H7* [26]. Parisi *et al.,* 2010 described lower prevalence of STEC 7 (5.7%) out of 123 raw milk samples in Apuila region [27]. However *E. coli O157: H7* was present in 39 (65%) out of sixty raw milk samples sold in Madurai city [28]. The occurrence of serotype *O157:H7* in milk and their products demonstrate to be protean in distinct localities as a result of variation in seasons, farm size, type of ration, hygiene, farm management practices, disparity in sampling, difference in the type of samples evaluated, and divergence in detection methods used [11,29].

The *E. coli O157:H7* pathogenicity is ascribed to production of shiga toxin genes *stx1*, *stx2* or combination of these toxins [30]. To investigate the presence of shiga toxin genes in isolates, mPCR was performed on all positive isolates of *0157:H7*. Twelve (12%) out of 100 isolates found positive for shiga toxin genes (*stx1* and *stx2*), 6/12 (50%) were found positive for *stx1*, 3/12 (25%) were found positive for *stx2* and 3/12 (25%) were found positive for both *stx1* and *stx2* genes as shown in **Table 4** and **Fig. 1**.

Our findings to some extent are in agreement with previous studies by Shahzad *et al.,* 2013 who reported 60 (73%) *stx1* and 51 (62%) *stx2* found in milk (raw) samples [20]. Studies conducted on milk have revealed current findings are in disagreement with some other previous studies who have reported higher frequency of *stx2* than *stx1* [31]. Studies conducted on bulk milk samples revealed 91% *stx2*, 8% *stx1* and 41% of both *stx1* and *stx2* genes [4]. The presence of stx2 gene demonstrated to be variable in distinct regions [27, 32].

In conclusion the present study revealed prevalence of *E. coli O157:H7* in raw milk from retail shops in Quetta. Shiga toxin genes of *E. coli O157:H7* (*stx1* and *stx2*) were identified in milk samples using mPCR. It is recommended that hygienic practices regulations should be instigated to promote safe and high quality of raw milk production.

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