Research Article

Isolation and Molecular Identification of Enterotoxigenic Strains of *Escherichia coli* in Raw and Pasteurized Milk

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Abstract | As milk is a staple food in human diet consequently the microbiological quality of milk is very important. Therefore, a study was carried out for the molecular characterization (identification) ETEC (Enterotoxigenic *Escherichia coli*) strains in both raw and pasteurized milk available in the market of District Kasur, Punjab (31.0896˚ N, 74.1240˚ E). A total of 65 samples of milk including 5 pasteurized milk samples from various sources were analyzed through scientific polymerase chain reaction (PCR) method including other microbiological laboratory techniques (Total plate count, Gram staining, Hydrogen sulphide, Citrate, Urease and Indole Tests). A high level of total plate count (TPC) and *E. coli* was observed from both raw and pasteurized milk. The results also indicated a high incidence of ETEC i.e. 63.63% and 50% in raw and pasteurized milk, respectively. The most occurring enterotoxins are ST-I and ST-II (40%) followed by LT-II and ST-II, 20% each, followed by ST-I (13.33%) and LT-I, LT-II (6.66%).

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Introduction

Pakistan is one of the largest agricultural country having a livestock population of 212.94 million heads, producing 63.684 million tons of milk annually (Economic Survey of Pakistan, 2020-2021). Up to 20% of milk is wasted due to non-availability of proper cooling and storage systems. Only 3% of milk finds its way to processing in urban market while the remaining 97% is consumed as raw milk, marketed through local milkmen. Milk has a distinct place among the foods used by human beings during

the first part of their lives. The studies show that animal products are complete and balanced food for the wellbeing of humans. History shows that milk and milk products are complete balanced diet for adults because it contains the entire essential nutrients, protein, fats, sugar, ash, and vitamins, needed for growth and development. It also supplies nutrients that would otherwise be difficult to obtain from food sources (Maijala, 2000). On the other hand, milk is also an ideal medium for the growth of microorganisms, both beneficial and pathogenic, the natural microflora of raw milk affects its quality characteristics. Microbial load is lower at the mammary tissues of a non-diseased, healthy animal with proper hygienic conditions during milking, handling, and processing which reduces chances of contamination as well as preserves the milk original characteristics (Fotou *et al*., 2011). Many factors are responsible for contamination of milk at farm level, these include farm demography, management, and cleanliness, hygiene of animals, milking techniques, and procedures (Elmoslemany *et al*., 2010). Before deciding on milk processing, the microbiological quality of the raw milk should be given importance at the dairy plant. This is called critical control point (CCP) in the hazard analysis critical control point (HACCP) plans. It should be enforced with in critical limits under the directives of the European Union law (Niza-Ribeiro *et al*., 2000). In a developing country like Pakistan, milk and its products are the main and important source of transmission of a large number of foodborne pathogens which poses a vital threat to human health. All these may be due to poor and unhygienic conditions of farms and poor animal health. *Escherichia coli* (*E. coli*) is one of the many and important species of bacteria living in the intestine of humans and animals. *E. coli*, based on virulence properties and toxin production, is divided into many groups. Enterotoxigenic *Escherichia coli* (ETEC) (Centers for Disease Control and Prevention, USA), verotoxigenic *Escherichia coli* (VTEC) is a well-recognized cause of hemolytic uraemic syndrome (HUS) in human beings, necrotoxigenic *E. coli* (NTEC-major cause of enteritis in animals), enteropathogenic *Escherichia coli* (EPEC) which known to cause diarrhea in humans, rabbits, dogs, cats, and horses, and enteroinvasive *Escherichia coli* (EIEC), found only in humans. The first group represents an important vector and a major cause of diarrhea in children of developing countries and traveler's diarrhea (Paneto *et al*., 2007). Enterotoxigenic *Escherichia coli* causes diarrhea by producing heat-labile enterotoxins (LT-I and LT-II), heat-stable enterotoxins (ST-I and ST-II), or both of these, by attaching to the intestinal mucosa, by their unique colonization factors (Nataro and Kaper, 1998). Previous data, in developing countries, shows an estimated cases of around 650 million and estimated deaths of 800,000 mostly in children. *Escherichia coli* detection in milk includes culture growth on selected media, biochemical tests, and serotyping of antibodies against specific bacterial antigens. These procedures are more difficult, cumbersome and more time-consuming as in some cases it takes several days to identify certain bacteria. Therefore, modern techniques should be followed as it can detect a small number of bacteria, their toxins and saves time and is more reliable. One of the modern techniques to test the presence of *E. coli* in milk and milk products is polymerase chain reaction (PCR), which is the most sensitive and widely used procedure for both identification and characterization of bacterial species (Hill, 1996; Wang *et al*., 1997). In Pakistan, poor farm hygienic conditions and transportation are not up to the mark so therefore we should thoroughly check the raw milk microbiology. We should give importance to *Coliforms*, *E. coli* which is of great public health concern. Therefore, study aims to find out enterotoxigenic *E. coli* (LT-I, LT-II, and ST-I, ST-II) through PCR to check its incidence in perspective of the milk quality.

Materials and Methods

Collection of samples

A total of 65 samples of raw milk were collected from commercial dairy farms, milk collection centers, and gawalas (milkman) (20 samples for each and 5 pasteurized milk samples). Each sample was collected in pre-sterilized screw-caped test tubes (50 ml) and properly marked for identification. Commercially available pouch packs of pasteurized milk samples, collected from the local market place, were also included in the study. All the samples were transported according to the set protocols of sample transportation.

Sterilization of glassware and media

All glassware were thoroughly cleaned, air-dried, and wrapped in paper for further sterilization in a hot air oven at 160°C for 2 hours (Alcamo, 1994). All the culture media used for the growth of bacteria in this research were properly sterilized by autoclaving at 121°C of 15 minutes at 15 pounds per square inch (PSI) pressure.

Laboratory procedures for microbial propagation
Following procedures were followed for all the samples for determination of total plate count (TPC) and *E. coli* count for further study.

Total plate count
Total plate count was performed according to the recommended method. The whole procedure was performed in sterilized laminar flow. Serial tenfold dilutions were prepared for each sample (Johnson and Case, 1995). To each test tube, a 9 ml phosphate buffer solution (PBS) was added. The test tubes were then sterilized by autoclaving. One ml of milk sample was added to the 1st tube with the help of a sterile pipette. From the 1st tube, 1 ml was added to the 2nd test tube and the same was repeated up to sixth test tube to make serial tenfold dilutions. Pre-autoclaved TPC media was cooled to 45°C and added to each Petri plate up to 25 ml approximately. One ml of the sample was transferred to Petri plate and was spread properly. The same was repeated for all dilutions. The Petri plates were marked accordingly to dilutions and were incubated for 24 hours at 37°C. After incubation colonies were counted by using a colony counter with a range from 20 to 200 colonies. The results were noted for TPC in terms of colony-forming unit per ml (CFU/ml).

E. coli count
MacConkey agar was used for culturing of *E. coli* in milk. The same pour plate technique was used as for TPC and the same serial dilutions were used. The MacConkey agar was autoclaved at 121°C for 15 minutes. After autoclaving, the media were cooled to 45°C and poured into Petri dishes, and was solidified. After solidification, the 1 ml sample was poured into Petri dishes and properly spread in plates. The plates were incubated at 37°C for 24 hours. Then the plates were selected for colony count which has the count between 20 and 200 colonies, CFU/ml was determined according to the following formula.

$$CFU/ml = No.\ of\ colonies \times Dilution\ factor$$

Isolation and purification of culture
To obtain pure culture of the organisms, one typical colony of *E. coli* was selected according to morphology and appearance and was further streaked on a MacConkey agar plate with the help of a sterilized loop in a laminar flow chamber. After streaking the plates were overnight incubated at 37°C. After the sub-culturing, the plates having visible colonies were selected for further microbiological testing.

Preservation of bacterial culture
Pure culture of the bacteria was transferred to MacConkey agar slant with the help of a sterilized loop by streaking. The slants were overnight incubated at 37°C, after incubation slants were refrigerated at 4°C.

Preliminary identification of the organism
Pure culture was used for preliminary identification of the organism by its morphological and biochemical profile.

Morphological examination
Typical *E. coli*, having pink in color, translucent, circular, smooth, and raised colony was selected and further examined by Gram staining.

Gram staining
Selected colonies were stained to be examined under a microscope. Gram staining was performed according to the recommended method.

Biochemical tests performed for further identification
Hydrogen sulphide test, Citrate test, Urease test and Indole test were performed according to the recommended methods of Winn *et al.* (2006) for further identification.

Preparation of culture for DNA extraction
The nutrient broth was prepared according to the manufacturer's instructions. The broth was autoclaved for 15 minutes at 121°C at 15 psi. The broth was cooled to 45°C and the culture was introduced to it with the help of a platinum loop. The broth was incubated at 37°C for 24 hours. After incubation, the turbidity in the broth showed the growth of cultured microorganisms. The tubes were kept under refrigerated temperature and later on used for DNA extraction. To avoid contamination control was kept without inoculation.

DNA extraction
DNA was extracted from overnight fresh broth culture as per the protocol of Paneto *et al.* (2007) was followed. 5 ml fresh broth culture was taken and centrifuged at 14000 rpm for 5 minutes, the supernatant was discarded and the pellets were suspended in 500 µl of distilled water and boiled

for 10 minutes and centrifuged again at 12000 rpm for 2 minutes. The supernatant of the centrifuged suspension was used for further analysis in PCR.

Polymerase chain reaction (PCR)

Genomic DNA was extracted from all the samples and PCR for all the *E. coli* positive samples was carried out. PCR amplification was done in 50µl volumes containing 5µl of template DNA, 200nM dNTPs, and 10mM of the respective primers, 1 U *Taq* DNA polymerase, and 5µl *Taq* buffer. Amplification of 16S DNA was carried out in 30 cycles (denaturation, 94°C for 30 sec, annealing (at primer respective temperature) for 2 min, and extension at 72°C for 5 minutes) in a thermocycler (Javed *et al.*, 2010). A control was used in each experiment to avoid the possibility of reagent contamination. Primers used were based on the known sequence from the available database NCBI Genbank. ETEC strains E 7476 (O166:H27; ST) and E 5798 (O7:H18; LT) were used as a positive control to check that the primers and PCR is working properly.

Agarose gel electrophoresis

Amplification of the thermocycler was confirmed by using 5µl of PCR product, mixed with 1µl of loading dye from each tube on 1.5-2.0 percent agarose gel (depending on the expected size of amplified product) at a constant voltage supply of 80-100V for 30min in single strength TBE buffer. Ethidium bromide was added at 5µl of 1% solution in 100 parts, gel solution in the gel. The product was visualized as a single compact fluorescent band of expected size under UV light and was documented by a gel documentation system (Bio-Rad, California USA).

Media used

All the media used in this study are Plate Count Agar, Macconkey Agar, Nutrient Broth, Kligler iron Agar, Simmons Citrate Agar, Urea Agar base, Tryptone Broth (Atlas, 2004) were procured from HiMedia Laboratories Pvt. Ltd. India.

Results and Discussion

The present project was designed to study the bacteriological quality of raw and pasteurized milk in summer months of June to August, regarding toxigenic *E. coli*. A total of sixty-five samples, twenty samples each from Commercial Dairy Farms (CDF), Milk Collection Centers (MCC), Gawalas (milkman), and five samples of pouch-packed pasteurized milk were collected and analyzed. All the samples were tested for total plate count and *E. coli* by using the pour plate method. Isolated organisms were identified by morphological and biochemical characteristics and polymerase chain reaction. The results are presented in the following tables.

Total plate count of the samples collected

All of the collected samples showed positive growth for total plate count when cultured microbiologically in Lab. Colonies were counted for the samples and were shown in the following tables.

Table 2 shows TPC for all the positive samples which range from 1.25×10^5 to 1.80×10^7 CFU/ml for commercial dairy farms, 2.10×10^5 to 2.00×10^7 CFU/ml for milk collection centers, 1.7×10^6 to 2.00×10^8 CFU/ml for Gawalas and 1.45×10^5 to 1.20×10^6 CFU/ml for pasteurized milk with standard deviation 6.4×10^6, 7.2×10^6, 8.0×10^7, 5.0×10^5 and mean 6.2×10^6, 7.6×10^6, 5.8×10^7 and 5.2×10^5 respectively. Most of the counts were in the range of 1,000,000 organisms per ml. No sample full filled the criteria for A-class raw milk as per OS standards.

Table 1: *Primers used in PCR amplification and their sequence is given in the table.*

Primer	Sequence	Product size (bp)	Annealing temp (°C)	Reference
LT-I	F: GGATTCATCATGCACCACAAGG R: CCATTTCTCTTTTGCCTGCCATC	360	63	Paneto *et al.* (2007)
LT-II	F: AGATATAATGATGGATATGTATC R: TAACCCTCGAAATAAATCTC	300	52	Paneto *et al.* (2007)
ST-I	F: TTTCCCCTCTTTTAGTCAGTCAACTG R: GGCAGGATTACAACAAAGTTCACAG	160	43	Pass *et al.* (2001)
ST-II	F: CCCCCTCTCTTTTGCACTTCTTTCC R: TGCTCCAGCAGTACCATCTCTAACCC	423	43	Pass *et al.* (2001)

Table 2: *Total plate count of all the samples of commercial dairy farms, milk collection centers, gawala, and pasteurized milk.*

Commercial dairy farms		Milk collection centers		Gawalas		Pasteurized milk	
Sample No.	Cfu/ml	Sample No.	Cfu/ml	Sample No.	Cfu/ml	Sample No.	Cfu/ml
1CDF	7.5×10^5	1MCC	8.0×10^6	1G	1.80×10^7	1PM	1.66×10^5
2CDF	8.0×10^5	2MCC	1.05×10^7	2G	2.20×10^7	2PM	1.45×10^5
3CDF	6.5×10^6	3MCC	1.08×10^7	3G	2.10×10^7	3PM	$1.20\ 10^6$
4CDF	6.3×10^5	4MCC	1.25×10^6	4G	1.80×10^8	4PM	9.5×10^5
5CDF	1.5×10^7	5MCC	1.45×10^7	5G	2.20×10^7	5PM	1.80×10^5
6CDF	1.10×10^6	6MCC	1.80×10^7	6G	1.80×10^7	-	-
7CDF	1.15×10^6	7MCC	1.90×10^6	7G	1.90×10^8	-	-
8CDF	1.25×10^7	8MCC	1.80×10^6	8G	2.00×10^7	-	-
9CDF	1.40×10^7	9MCC	2.10×10^5	9G	1.80×10^7	-	-
10CDF	1.80×10^7	10MCC	1.90×10^7	10G	1.87×10^6	-	-
11CDF	1.60×10^7	11MCC	1.80×10^7	11G	1.86×10^6	-	-
12CDF	1.66×10^6	12MCC	2.00×10^7	12G	1.90×10^7	-	-
13CDF	1.25×10^5	13MCC	1.70×10^6	13G	2.10×10^8	-	-
14CDF	1.84×10^6	14MCC	1.60×10^6	14G	2.15×10^7	-	-
15CDF	7.5×10^5	15MCC	1.35×10^7	15G	2.05×10^7	-	-
16CDF	7.8×10^5	16MCC	1.45×10^6	16G	1.70×10^6	-	-
17CDF	9.0×10^5	17MCC	1.60×10^6	17G	1.85×10^8	-	-
18CDF	9.5×10^6	18MCC	1.10×10^5	18G	2.00×10^8	-	-
19CDF	8.0×10^6	19MCC	8.0×10^6	19G	1.90×0^6	-	-
20CDF	1.45×10^7	20MCC	1.20×10^6	20G	1.90×10^6	-	-
Mean	6.2×10^6	Mean	7.6×10^6	Mean	5.8×10^7	Mean	5.2×10^5
Std. Dev	6.4×10^6	Std. Dev	7.2×10^6	Std. Dev	8.0×10^7	Std. Dev	5.0×10^5
Mini.	1.25×10^5	Mini.	1.10×10^5	Mini.	1.70×10^6	Mini.	1.45×10^5
Max.	1.80×10^7	Max.	2.00×10^7	Max.	2.10×10^8	Max.	1.20×10^6

**CFU: colony-forming unit per ml.*

Table 3 shows that 7 samples out of 20 (35%) were *E. coli* positive for commercial dairy farms, 5 out of 20 (25%) for milk collection centers, 8 out of 20 (40%) for gawalas, and 2 out of 5 (40%) were positive for pasteurized milk. The table also shows that 33.34% of samples were positive for *E. coli*.

Table 3: *Total E. coli positive samples out of total collected samples.*

Source	No. of samples	*E. coli* +ive	Percentage %
Commercial dairy farms	20	07	35
Milk collection centers	20	05	25
Gawalas (milkman)	20	08	40
Pasteurized milk	05	2	40
Total	65	22	33.34

Table 4 shows *E. coli* positive samples (CFU/ml). *E. coli* count were ranged from 8.5×10^5 to 9.0×10^6 CFU/ml for commercial dairy farms samples, 1.15×10^5 to 9.0×10^7 CFU/ml, 1.30×10^5 to 1.90×10^8 CFU/ml, and 8.5×10^6 to 1.20×10^7 CFU/ml for milk collection centers, gawalas, and pasteurized milk respectively. The results showed a higher *E. coli* load in the samples which indicates heavy post milking contamination.

All the *E. coli* positive samples were further subjected to biochemical tests. Indole, catalase, urease, and hydrogen sulfide production tests were performed for the positive samples which are shown in the above Table 5. All the tests results confirmed the presence of *E. coli* in all the samples.

Table 6 shows the PCR result of all the *E. coli* positive samples of all four sources. All the *E. coli* positive samples were amplified through PCR to detect the enterotoxigenic strains.

Table 4: *E. coli count of commercial dairy farms, milk collection centers, gawala, and pasteurized milk.*

Commercial dairy farms		Milk collection centers		Gawalas		Pasteurized milk	
Sample No.	Cfu/ml	Sample No.	Cfu/ml	Sample No.	Cfu/ml	Sample No.	Cfu/ml
3CDF	8.5×10^{5}	4MCC	9.0×10^{7}	2G	1.40×10^{7}	1PM	8.5×10^{6}
4CDF	1.20×10^{6}	9MCC	1.10×10^{7}	5G	1.55×10^{8}	4PM	1.20×10^{7}
7CDF	1.35×10^{6}	13MCC	1.20×10^{6}	6G	1.60×10^{7}	-	-
11CDF	9.0×10^{6}	14MCC	1.45×10^{6}	9G	1.30×10^{5}	-	-
15CDF	8.3×10^{5}	17MCC	1.15×10^{5}	12G	1.85×10^{6}	-	-
19CDF	1.13×10^{6}	-	-	15G	1.80×10^{8}	-	-
20CDF	8.0×10^{6}	-	-	16G	1.70×10^{7}	-	-
-	-	-	-	19G	1.90×0^{8}	-	-
Mean	3.1×10^{6}	Mean	2.07×10^{7}	Mean	7.1×10^{7}	Mean	6.4×10^{6}
Std. Dev	3.6×10^{6}	Std. Dev	3.8×10^{7}	Std. Dev	8.6×10^{7}	Std. Dev	7.8×10^{6}
Mini.	8.3×10^{5}	Mini.	1.15×10^{5}	Mini.	1.30×10^{5}	Mini.	8.5×10^{5}
Max.	9.0×10^{6}	Max.	9.0×10^{7}	Max.	1.90×10^{8}	Max.	1.20×10^{7}

Table 5: *Biochemical tests for isolated E. coli.*

Source	Sample no.	Biochemical tests			
		Indole	Citrate	Urease	H_2S
Commercial dairy farms	3CDF	+	-	-	-
	4CDF	+	-	-	-
	7CDF	+	-	-	-
	11CDF	+	-	-	-
	15CDF	+	-	-	-
	19CDF	+	-	-	-
	20CDF	+	-	-	-
Milk collection centers	4MCC	+	-	-	-
	9MCC	+	-	-	-
	13MCC	+	-	-	-
	14MCC	+	-	-	-
	17MCC	+	-	-	-
Gawalas	2G	+	-	-	-
	5G	+	-	-	-
	6G	+	-	-	-
	9G	+	-	-	-
	12G	+	-	-	-
	15G	+	-	-	-
	16G	+	-	-	-
	19G	+	-	-	
Pasteurized milk	1 PM	+	-	-	-
	4 PM	+	-	-	-

Table 6: *Enterotoxigenic E. coli positive samples.*

Source	Samples no.	LT-I	LT-II	ST-I	ST-II
Commercial dairy farms	3CDF	-	-	+	+
	4CDF	-	+	-	-
	7CDF	-	-	+	-
	11CDF	-	-	-	+
	15CDF	-	-	-	-
	19CDF	-	-	-	-
	20CDF	-	-	-	-
Milk collection centers	4MCC	-	-	-	-
	9MCC	-	-	-	+
	13MCC	-	-	+	+
	14MCC	-	-	+	-
	17MCC	-	-	-	+
Gawalas	2G	-	-	-	-
	5G	-	-	+	+
	6G	-	+	-	-
	9G	+	+	-	-
	12G	-	-	-	+
	15G	-	-	+	+
	16G	-	-	+	+
	19G	-	-	-	-
Pasteurized milk	1 PM	-	-	+	+
	4 PM	-	-	-	-
Total	22	1	3	8	10

Table 7 shows the percentage of the presence of enterotoxin in all the *E. coli* positive isolates from all the sources. The table also shows the percentage of the presence of all the four individual strains or their combined presence.

Figure 1 shows the presence of the LT-I strain in all the *E. coli* positive samples. The figure shows that only one sample is positive for the presence of LT-I strain of enterotoxigenic strain of *E. coli*. Lane 1 represents

sample number 9G for gawala milk while the rest of the samples were negative for LT-I.

Table 7: *Percentage (%) of the enterotoxins in the positive E. coli samples.*

Source*	*E. coli* +ve isolates	Enterotoxin positive	LT-I	LT-II	ST-I	ST-II	LT-I, LT-II	ST-I, ST-II
CDF	07	57	-	25	25	25	-	25
MCC	05	80	-	-	25	50	-	25
G	08	75	-	33.33	-	-	16.66	50
PM	02	50	-	-	-	-	-	100
Total	22	68	-	20	13.33	20	6.66	40

**CDF: commercial dairy farms; MCC: milk collection centers; G: gawalas; PM: pasteurized milk.*

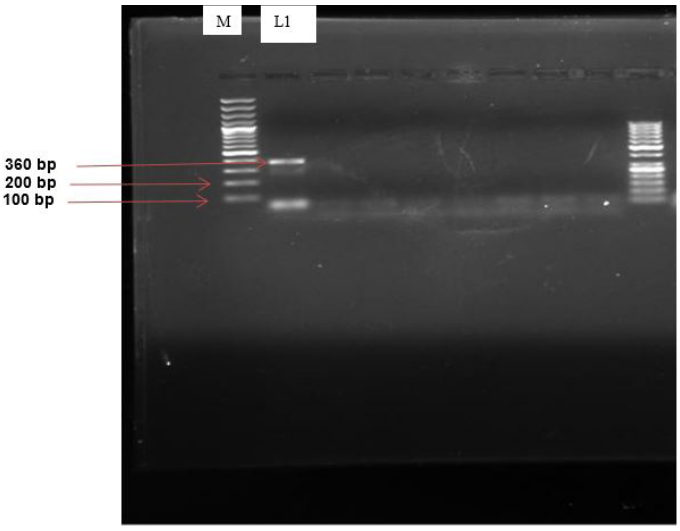

Figure 1: *PCR amplified result (gel electrophoresis) for LT-I in all E. coli positive samples.*

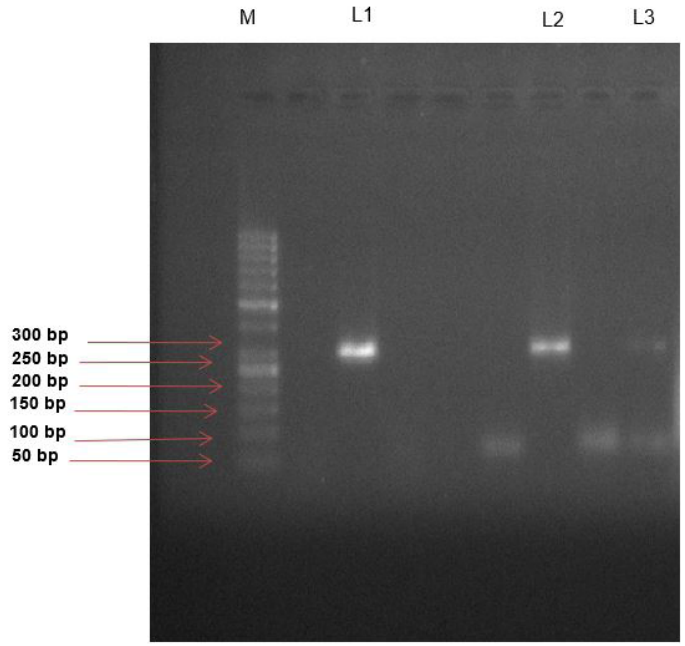

Figure 2: *PCR amplified result (gel electrophoresis) for LT-II in all E. coli positive samples.*

Figure 2 shows the presence of an LT-II strain in all the *E. coli* positive samples. The figure indicates that three samples are positive for the presence of the LT-II strain of enterotoxigenic *E. coli*. Lane 1 represents sample number 4CDF of commercial dairy farms, lanes number 2 and 3 represent sample numbers 6G and 9G of gawala while all other samples were negative for LT-II.

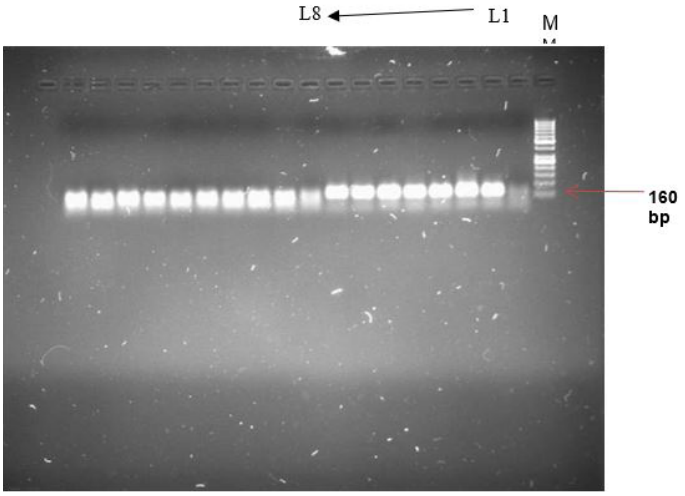

Figure 3: *PCR amplified result (gel electrophoresis) for ST-I in all E. coli positive samples.*

Figure 3 shows the presence of the ST-I strain in all the *E. coli* positive samples. The figure indicates that 8 samples are positive for the presence of ST-I strain of enterotoxigenic *E. coli*. Lane 1 and 2 represent sample numbers 3CDF and 7CDF of commercial dairy farms, lanes 3 and 4 represent sample numbers 13MCC and 14MCC of milk collection centers, and lanes number 5, 6, and 7 represent samples number 5G, 15G, and 16G of gawalas, respectively. Lane 8 represents sample number 1 PM of pasteurized milk while all other samples were negative for the presence of ST-I.

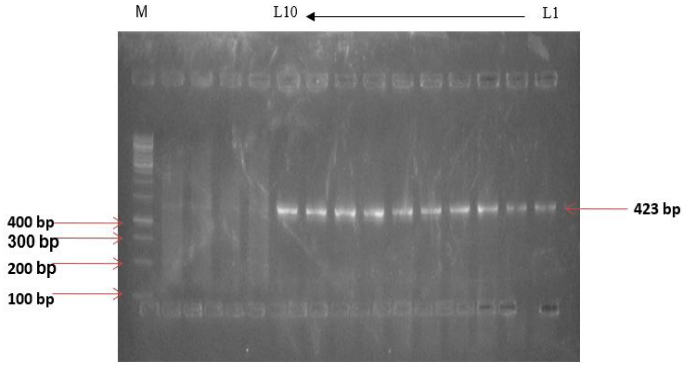

Figure 4: *PCR amplified result (gel electrophoresis) for ST-II in all E. coli positive samples.*

Figure 4 shows the presence of the ST-II strain in

all the *E. coli* positive samples. The figure indicates that ten samples are positive for the presence of the ST-II strain of enterotoxigenic *E. coli*. Lane 1 and 2 represent sample numbers 3CDF and 11CDF of commercial dairy farms. Similarly, lanes 3, 4, and 5 represent sample number 9MCC, 13MCC, and 17MCC of milk collection centers, lanes 6, 7, 8, 9, and 10 represent sample numbers 5G, 12G, 15G, 16G, and 1 PM of gawala and pasteurized milk respectively while all other samples were negative for ST-II.

The present study was conducted to evaluate the quality of raw milk in the summer months (June-August) concerning microbial growth and the incidence of toxigenic *E. coli*. Total plate count and *E. coli* count were determined for all the samples. Further, an attempt was made to identify toxigenic strains of *E. coli* through a polymerase chain reaction. The total plate count (TPC) of the raw and pasteurized milk ranged from 1.25×10^5 to 2.00×10^8 CFU/ml. Mutukumira *et al.* (1996) found TPC between 6.2×10^3 and 7.78×10^7 CFU/ml of raw milk. Similarly, Stojanovic (1994) found a higher load between $6x10^4$-$2.4x10^8$ CFU/ml. However, Yoo *et al.* (1994) found a lower plate count of $8.3x10^4$- $4.0x10^5$ CFU/ml. Also, Kashifa (2000) found a lower plate count of $6.9x10^3$-$1.12x10^7$ CFU/ml. None of the sample were of good quality. TPC also showed a variety of spoilage, LAB and pathogenic bacteria (Nangamso, 2006; Quinn *et al.*, 2002; Bonsu *et al.*, 2000; Weinhaupl *et al.*, 2000) and reflects milking hygiene. Cleaning of the udder with water, hand milking, and milking utensils play an important role as a contaminant to milk (Filipoviet and Kokaj, 2009). Some farms are located far away from collection centers and hence more distance from the collection centers contributes to the higher count of bacteria (Mutukumira *et al.*, 1996). As milk is favorable medium for microorganisms which favorably grows at a temperature above 16°C and most of our farmers don't have a cooling facility and electricity shortage is a common problem over here so milk is exposed for a longer time to ambient temperature. The possibility of such milk containing pathogenic bacteria like *Brucella* spp., *Mycobacterium Bovis*, *Salmonella* spp., *Listeria monocytogenes,* and *Campylobacter jejuniwhich* are capable of causing different types of milk-borne illnesses in humans which cannot be completely ruled out (Kumbhar *et al.*, 2009; Nanu *et al.*, 2007). *E. coli* are gram-negative microorganisms that ferment lactose. They are important in routine examination of milk as their presence indicates unhygienic conditions at the cowshed and dairy farm hence represents post milking contamination. Out of 65 samples, 22 (33.84%) were positive for *E. coli* presence which is very less than 66% as reported by Altalhi and Hassan (2009). In the present study *E. coli* count in raw and pasteurized milk was $1.15x10^5$–$1.90x10^8$ CFU/ml and $8.5x10^6$-$1.2x10^7$ CFU/ml respectively. Mishra and Kulla (1989) observed a much lesser count of $6.5x10^3$ CFU/ml of raw milk. Hamama and El-mouktaktafi (1990) found a closer figure of $1.8x10^5$ CFU/ml whereas Stanescu *et al.* (1992) also found a closure figure of $3.8x10^5$ CFU/ml. The finding of this study is much alarming regarding the hygienic and sanitary conditions of our raw milk supplies. Pasteurized milk samples also show a very high *E. coli* count. Stanescu *et al.* (1992) also found a higher *E. coli* count of $1.34x10^5$ CFU/ml. Similar to TPC the high count of *E. coli* in raw and pasteurized milk shows low level of milking hygiene and high post milking contamination (Abdel-all and Dardir, 2009). When pasteurization is correctly done it is presumed that the level of heat-sensitive bacteria is reduced in milk (Gran *et al.*, 2003). Therefore, the high level of *E. coli* in this study should be due to environmental and post-pasteurization contamination. The *E. coli* positive samples from all four sources were subjected to a polymerase chain reaction (PCR) for the detection of enterotoxigenic *E. coli* strains. The PCR results show that 15 out of 22 isolates (68%) were positive for enterotoxigenic *E. coli* which is less than 96% of the study carried out by Paneto *et al.* (2007) but close to 66.66% find out by Osek (2001). A similar result of 69.7% was found out by Altalhi and Hassan (2009). A close result was also reported by Patil *et al.* (1999) of 75% presence of enterotoxigenic strains. Out of 15 enterotoxigenic positive samples, 3 (20%) were positive for the LT-II strain. Similarly, Paneto *et al.* (2007) also reported 15% LT-II present in raw milk and its products. Two isolates (13.33%) were positive for heat-stable enterotoxin ST-I strain which is higher than 3.9% reported by Salvadori *et al.* (2003). Three isolates (20%) were positive for the presence of heat-stable (ST-II) strain. Altalhi and Hassan (2009) reported a 6.1% presence of ST-II strain in milk. One isolate (6.66%) was positive for both LT-I and LT-II and six isolates (40%) were positive for both ST-I and ST-II strains. More enterotoxigenic strains (80%) were found in milk collections centers. Similarly, 75%, 57%, and 50% enterotoxigenic strains were found in Gawala milk, commercial dairy farms, and pasteurized milk, respectively. In conclusion,

it is observed in the present study that due to unhygienic farms conditions, poor sanitation system, non-hygiene milking conditions, and post milking and pasteurization contamination there is a high bacterial count both in raw and pasteurized milk. The study also shows that due to the above-mentioned conditions along with high bacterial count there is a high *E. coli* count was also observed. The hot weather and dirty environment also play a key role in the high *E. coli* count. The results also show that among the *E. coli* positive samples ST-I, ST-II combined are the most common enterotoxins. The other most common enterotoxins are LT-II (20%), ST-II (20%), ST-I (13.33%), and LT-I, LT-II combined (6.66%).

Conclusions and Recommendations

The present study indicates a high level of presence of total plate count and *E. coli* count at the rate of LT-II (20%), ST-II (20%), ST-I (13.33%), and LT-I, LT-II combined (6.66%). The study also shows that the incidence of enterotoxigenic *E. coli* which is very high both in raw and pasteurized milk. It is concluded that this high level of incidence is mainly due to improper management, unsanitary farm conditions, unhygienic milk production, cleanness of milking equipment, and post-pasteurization contamination. An unhygienic environment also plays a very important role in the contamination of milk whether it's raw or pasteurized.

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Novelty Statement

This is a novel approach and first study in Pakistan to observe enterotoxigenic *E. coli* in milk for the molecular identification of enterotoxigenic *Escherichia coli* (ETEC) strains in raw and pasteurized milk of Punjab, District Kasur.

Author's Contribution

Rahman Ullah, Muhammad Junaid and Nabila Gulzar: Conceived, designed and carried out the experiment.
Rahat Ullah Khan, Mushtaq Ahmed and Baseer Ahmad: Analyzed the data.
Ambrina Tariq, Aamir Iqbal and Mirwaise Khan: Wrote, organized the data and materialized the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

References

Abdel-All, A.A.A., and H.A. Dardir. 2009. Hygienic quality of local traditional fermented skimmed milk (laban rayb) sold in Egypt. World J. Dairy Food Sci., 4(2): 205-209.

Alcamo, I.E., 1994. Fundamentals of microbiology, 4th Ed. Benjamin Commings Publishing Company Inc. pp. 645-646.

Altalhi, A.D. and S.A. Hassan. 2009. Bacterial quality of raw milk investigated by *Escherichia coli* and isolates analysis for specific virulence-gene markers. Food Contr., 20(10): 913-917.

Atlas, R.M., 2004. Hand book of microbiology media 3rd Ed. CRC Press, New York. https://doi.org/10.1201/9781420039726

Bonsu, O.A, E. Laing, and B.D. Akanmori. 2000. Prevalence of tuberculosis in cattle in the Dangme-West district of Ghana, public health implications. Acta Trop., 76: 9-14. https://doi.org/10.1016/S0001-706X(00)00082-6

CDC (Centers for Disease Control and Prevention). 2021. Enterotoxigenic E. Coli. Accessed at https://www.cdc.gov/ecoli/etec.html#:~:text=Enterotoxigenic%20Escherichia%20coli%2C%20or%20ETEC,excessive%20fluid%2C%20thus%20producing%20diarrhea

Cocolina, L., G. Astoria, M. Manzanoa, C. Cantonib and G. Comi. 2000. Development and evaluation of a PCR–microplate capture hybridization method for direct detection of verotoxigenic *Escherichia coli* strains in artificially contaminated food samples. Int. J. Food Microbiol., 54: 1–8. https://doi.org/10.1016/S0168-1605(99)00169-5

Economic Survey of Pakistan, 2020-2021.

Government of Pakistan. 2020-2021

Elmoslemany, A. Morsy, G.P. Keefe, I.R. Dohoo, J.J. Wichtel, H. Stryhn and R.T. Dingwell. 2010. The association between bulk tank milk analysis for raw milk quality and on-farm management practices. Prevent. Vet. Med., 95(1-2): 32-40.

Filipoviet, D., and M. Kokaj. 2009. The comparison of hand and machine milking on small family dairy farms in Central Croatia. Livest. Res. Rural Dev., 21: 5-8.

Fotou, K., A. Tzora, C. Voidarou, A. Alexopoulos, S. Plessas, I. Avgeris, E. Bezirtzoglou, K. Akrida-Demertzi, and P.G. Demertzis. 2011. Isolation of microbial pathogens of subclinical mastitis from raw sheep's milk of Epirus (Greece) and their role in its hygiene. Anaerobe, 17: 315-319. https://doi.org/10.1016/j.anaerobe.2011.05.002

Giannino, M.L., M. Aliprandi, M. Feligini, L. Vanoni, M. Brasca and F. Fracchetti. 2009. A DNA array based assay for the characterization of microbial community in raw milk. J. Microbiol. Methods, 78: 181-188. https://doi.org/10.1016/j.mimet.2009.05.015

Gran, H.M., T.H. Gadaga and J.A. Narvhus. 2003. Utilization of various starter cultures in the production of amasi, a Zimbabwean naturally fermented raw milk product. Int. J. Food Microbiol., 88: 19-28. https://doi.org/10.1016/S0168-1605(03)00078-3

Hamama, A., and M. El-Mouktaktafi. 1990. Study of the hygienic quality of raw milk produced in morocco. Mahgreb-Veterinaire, 5: 17-20.

Hemraj, V., S. Diksha and G. Avneet. 2013. A review on commonly used biochemical tests for bacteria. Innov. J. Life Sci., 1: 1-7.

Hill, W.E., 1996. The polymerase chain reaction: application for the detection of food borne pathogen. Crit. Rev. Food Sci. Nutr., 36: 123-173. https://doi.org/10.1080/10408399609527721

Javed, I., S. Ahmad, M.I. Ali, B. Ahmad, P.B. Ghumro, A. Hameed and G.J. Chaudry. 2010. Bacteriocinogenic potential of newly isolated strains of *Enterococcus faecium* and *Enterococcus faecalis* from dairy products of Pakistan. J. Microbiol. Biotechnol., 20: 153-160. https://doi.org/10.4014/jmb.0904.04024

Johnson, R.T., and C.L. Case. 1995. A laboratory experiment in microbiology, 4th Ed. The Benjamin-Cummings Publishing Company Inc. pp. 36-62.

Kashifa, K., 2000. Bacteriological studies on the raw milk supplied to Faisalabad city during summer months. M. Sc. thesis, Department of Vet. Microbiology, Faculty of Vet. Sciences, University of Agriculture, Faisalabad.

Kumbhar, S.B., J.S. Ghosh and S.P. Samudre. 2009. Microbiological analysis of pathogenic organisms in indigenous fermented milk products. Adv. J. Food Sci. Tech., 1: 35-38.

Laura, C.R.L.C., J. Bai, A.J. Martinez, M.C. Vanegasa and O.G. Gomez-Duarte. 2010. Molecular characterization of diarrheagenic *Escherichia coli* strains from stools samples and food products in Colombia. Int. J. Food Microbiol., 138: 282-286. https://doi.org/10.1016/j.ijfoodmicro.2010.01.034

Maijala, K. 2000. Cow milk and human development and well-being. Livest. Prod. Sci., 65(1-2): 1-18. https://doi.org/10.1016/S0301-6226(00)00194-9

Mishra AK, Kulla RK. 1989. Bacteriological quality of market milk. Ind Dairyman. 41: 487-491.

Mutukumira, A.N., S.B. Feresu, J.A. Narvhus and R.K. Abrahamsen. 1996. Chemical and microbiological quality of raw milk produced by smallholder farmers in Zimbabwe. J. Food Prot., 59: 984-987. https://doi.org/10.4315/0362-028X-59.9.984

Nangamso, B.C., 2006. General hygiene of commercially available milk in the Bloemfontein Area (Unpublished MSc Thesis, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa.

Nanu, E., C. Latha, B. Sunil, T.M. Prejit and K.V. Venon. 2007. Quality assurance and public health safety of raw milk at the production point. Am. J. Food Tech., 2: 145-152. https://doi.org/10.3923/ajft.2007.145.152

Nataro, J.B., and J.B. Kaper. 1998. Diarrheagenic *Escherichia coli.* Clin. Microbiol., 11: 142-201. https://doi.org/10.1128/CMR.11.1.142

Niza-Ribeiro, J., A.C. Louza, P. Santos, and M. Lima. 2000. Monitoring the microbiological quality of raw milk through the use of an ATP bioluminescence method. Food Contr., 11: 209-216. https://doi.org/10.1016/S0956-7135(99)00101-2

Osek, J. 2001. Multiplex polymerase chain reaction assay for identification of enterotoxigenic *Escherichia coli* strains. J. Vet. Diagn. Invest.,

13(4): 308-311.

Paneto, B.R., R.P. Schocken-Iturrino, C. Macedo, E. Santo and J.M. Marin. 2007. Occurrence of toxigenic *Escherichia coli* in raw milk cheese in Brazil. Arq. Bras. Med. Vet. Zoot., 59: 508-512. https://doi.org/10.1590/S0102-09352007000200035

Patil, R.M., A.G. Karpe and M.B. Gujar. 1999. Studies on drug resistance and virulence of *Escherichia coli* from diarrheic calves. Indian J. Comp. Microbiol Immun. Infect. Dis., 20: 29-31.

Quinn, P.J., M.E. Carter, B. Markey and G.R. Carter. 2002. Clinical veterinary microbiology. Grafos, Spain.

Salvadori, M.R., G.F. Valadares, D.S. Leite, J. Blanco and T. Yano. 2003. Virulence factors of *Escherichia coli* isolated from calves with diarrhea in Brazil. Braz. J. Microbiol., 34: 230-235. https://doi.org/10.1590/S1517-83822003000300009

Stanescu, V., F. Chirila, C. Sahleanu, V. Vana and A. Damian. 1992. The level of coliform and *E. coli* titer as hygienic quality index of raw and pasteurized milk and chicken meat. Proceedings of the 3rd World Congress on Foodborne Infections and Intoxications, Berlin, Germany. pp. 16-19.

Steel, R.G.D., J.H. Torrie and S.A. Dickey. 1997. Principles and procedures of statistics. A biometrical approach 3rd Edn. McGraw Hill Book Co. New York.

Stojanovic, E., 1994. The total number of microorganisms in raw milk. Vet. Glasnik, 48: 291-294.

Wang, R.F., W.W. Cao and C.E. Cerniglia. 1997. A universal protocol for PCR detection of 13 species of food borne pathogens in foods. J. Appl. Microbiol., 83: 727-736. https://doi.org/10.1046/j.1365-2672.1997.00300.x

Weinhaupl, I., K.C. Schopf, D. Khaschabi, A.M. Kapaga and H.M. Msami. 2000. Investigations on the prevalence of bovine tuberculosis and brucellosis in dairy cattle in Dar es Salaam region and Zebu cattle in Lugoba area, Tanzania. Trop. Anim. Health Prod., 32: 147-154. https://doi.org/10.1023/A:1005231514467

Winn, W., S. Allen, W. Janda, E. Koneman, G. Procop, P. Schreckenberger and G. Woods. 2006. Color atlas and textbook of diagnostic microbiology, 6th Ed. Lippincott Williams and Wilkins, Philadelphia, PA. pp. 224-226.

Yoo, S.A., W.M. Jeon, S.H. Kim and Y.K. Kim. 1996. Effect of various factors in farm management on raw milk quality. Korean J. Dairy Sci., 18: 229-236.

Yu, G., J. Niu, M. Shen, H. Shao and L. Chen. 2006. Detection of *Escherichia coli* O157 using equal-length double-stranded fluorescence probe in a real-time polymerase chain reaction assay. Clin. Chim. Acta, 366: 281-286. https://doi.org/10.1016/j.cca.2005.10.027