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ERIC-PCR Based Molecular Typing of *Escherichia coli* Recovered from Milk in Peshawar, Pakistan

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FK performed samples collection, experimental work, data analysis and manuscript writing. KA conceived the idea, contributed to experimental work, conducted analysis, contributed to manuscript writing and proofreading. MJU and SH helped in data analysis and manuscript writing. All the authors approved the final version of the manuscript.

Key words
ERIC-PCR, *E. coli*, Genetic diversity, Milk, Public health, Contamination

ABSTRACT

Milk is frequently contaminated with *Escherichia coli* that could cause infections in human through the food chain. This study aimed to investigate genetic diversity among *Escherichia coli* isolates recovered from milk samples collected from shops in Peshawar, Pakistan, using Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR). A total of 300 milk samples were collected and processed for isolation of *E. coli*. Two hundred *E. coli* isolates were recovered from these milk samples that were confirmed through morphological and biochemical tests. All the isolates were subjected to ERIC-PCR which generated distinct banding pattern indicative of high genetic diversity. The data generated was subjected to further phylogenic analysis. This analysis revealed 98 clusters of *E. coli* based on a similarity coefficient of ≥85%. Isolates from the same or adjoining areas were found to have high genetic similarity showing the clonal dissemination of these isolates. In contrast, isolates from different areas showed a substantially high genetic diversity that suggests multiple routes of milk contamination. The main contributing factors to this multi-source contamination could be inadequate hygiene, inefficient equipment cleaning, suboptimal milk handling and suboptimum storage conditions. These findings highlight the need for development of proper sanitary conditions and quality assurance measures for production and distribution of milk to reduce *E. coli* contamination and to mitigate the resulting public health hazard. The study also highlights the usability of ERIC-PCR for estimating genetic diversity in *E. coli* of milk origin. This method could supplement microbiological and surveillance studies for devising better control and management measures especially in low income countries.

INTRODUCTION

Milk is an important source of food that contains essential nutrients and is globally used to meet daily nutritional requirements. Raw milk directly coming out from the udder of healthy animals is considered safe for human use because it contains fewer microorganisms. However, it can be instantly contaminated through transfer of bacteria from animal feces, workers, surroundings (air, soil, milking equipment, water), dirty exterior surfaces of udders, etc. (Deddefo *et al*., 2023). The quality of products across the dairy chain is determined by the initial microbial load of the raw milk at the farm level. Thus, it is important to follow proper hygiene practices during handling of milk at the farm level (Chimuti *et al*., 2024). One contaminant of major importance is *Escherichia coli* that is frequently associated with foodborne diseases.

E. coli is common inhabitant of warm blooded animals including human lower intestine. It is released into the environment through feces that could easily contaminate water and food. It is also frequently used as an indicator to assess the quality of water and food (Jang *et al*., 2017). Many strains of *E. coli* are harmless, but some, such as Shiga toxin-producing *E. coli* (STEC), could cause serious diseases. The main source of *E. coli* infections in humans with this bacterium is contaminated food such as beef, raw milk, vegetables, and fruits. Ruminants are natural reservoirs for STEC strain. Contamination of milk with this strain of *E. coli* is frequent. *E. coli* is also a common inhabitant of soil, water, and animal bedding (Mesele *et al*., 2023). The vicinity of anus to the udder is another source of milk contamination with *E. coli* (Calahorrano-Moreno *et al*., 2022). *E. coli* is also one of the causes of mastitis that is a devastating disease in dairy cattle. It easily spreads from diseased animals to healthy animals through milking equipment and workers (Goulart and Mellata, 2022). These are potential sources of milk contamination at the farm level. Other factors involved in milk contamination

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include inadequate temperature treatment and improper packaging, storage, and transportation (Berhe *et al*., 2020). Once milk is contaminated at any level of the chain, it poses serious health risks to consumers. Most bacteria can be killed by heating raw milk, but toxins are heat resistant and pose a risk to end users (Owusu-Kwarteng *et al*., 2020). Thus, it is important to avoid contamination of milk with bacteria at each level of processing.

To reduce bacterial contamination in food, it is important to trace the main sources of contamination of *E. coli*. One efficient and reliable way to perform this tracing is to analyze bacterial genomes to group them on the basis of ecological niches. The bacterial genome contains repetitive sequences that could be accessed to determine genetic similarity and variability among bacterial strains. The frequency and location of these repeats varies among bacterial strains. This variation is the basis for determining clonal relatedness among isolates (Subirana and Messeguer, 2020). Various molecular methods that utilize these sequences to identify the sources of microbial contamination are available. Among these, the molecular technique of Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) is simple, easy to perform, less time-consuming and inexpensive (Ranjbar *et al*., 2016). Enterobacterial repetitive intergenic consensus (ERIC) repeats are imperfect palindromes, 122-127 bp in size and their copy numbers varies among bacterial strains (Bakhshi *et al*., 2018). They were first identified in *E. coli, Salmonella*, and other Enterobacteriaceae members (Bakhshi *et al*., 2018). The difference in their numbers among different bacterial isolates is used as a tool for genetic screening (Otokunefor *et al*., 2020).

In the present study, ERIC-PCR was used to estimate genetic variability among *E. coli* isolates recovered from milk in Peshawar, Pakistan. The study attempted to figure out the sources of milk contaminating *E. coli* in the region by determining genetic relatedness among the *E. coli* isolates from various locations. This information is crucial for devising proper strategies for ensuring milk safety for consumers and equipping the health sector to better respond to future outbreaks of *E. coli* infections.

MATERIALS AND METHODS

Sampling

A total of 300 milk samples (30 ml) were collected from different retail centers in Peshawar, Pakistan. Sterile falcon tubes were used to collect the samples. The samples were immediately transported to the laboratory at Center of Biotechnology and Microbiology, University of Peshawar and were processed directly or refrigerated until future analysis.

Isolation and identification of E. coli

For isolation of *E.coli*, each milk sample was diluted in peptone buffered water up to 10^{-5}. Each diluted sample was inoculated onto MacConkey ager media plate using spread plate method. Plates were incubated at 37°C for 24 h. After incubation, colonies producing characteristic metallic sheen color were considered as presumptive *E.coli* (Younis *et al*., 2021). All isolates were subsequently analyzed through microscopic and biochemical tests. Which included Gram staining, catalase, oxidase, methyl red, Voges-Proskauer, citrate utilization, triple sugar iron and indole tests (Megersa and Abdelhamid, 2019).

DNA isolation

All *E. coli* isolates were processed for genomic DNA extraction using the GeneJet Genomic (Catalog No. FERK0721) Bacterial DNA extraction Kit. Gel electrophoresis was afterwards performed to verify the quality of extracted DNA. DNA was stored at -20 °C for subsequent use.

ERIC-PCR

A total of 200 recovered *E. coli* isolates were subjected to Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) fingerprinting using reported primers (Versalovic *et al*., 1991). Reaction mixture (20 µL) consisted of molecular grade water (13 µL), Master Mix (4 µL, Solis BioDyne, Estonia, Catalogue No. 04-12-00115, containing Taq polymerase, reaction buffer, dNTPs and $MgCl_2$), reverse (5′AAGTAAGTGACTGGGGTGAGCG3′) and forward (5′ATGTAAGCTCCTGGGGATTCAC3′) primers (1 µL each, primer) and template DNA (1 µL). Amplification conditions were: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturing at 90 °C for 30 seconds, annealing at 40 °C for 1 min, extension at 72 °C for 1 min and a final denaturing at 72 °C for 8 min. Products of PCR were resolved using a 1.5% agarose gel and 100 bp ladder (Solis BioDyne, Catalogue No. 07-11-00050). After electrophoresis results were analyzed through gel documentation system. All easily observable and reproducible bands (also termed as Alleles) in each well were recorded according to the molecular sizes. This data resulting for the gel electrophoresis banding pattern was used for further statistical analysis.

Statistical and bioinformatics analysis

To estimate genetic diversity, the ERIC-PCR profiles for all isolates were subjected to statistical and bioinformatics analyses to generate bivariate data and

calculate genetic distances among the isolates. For this purpose, presence of an allele (band) was marked as 1 and absence of the the allele was represented as 0. To calculate genetic distances, the formula Dxy = 1– {(Nxy) / (Nx + Ny – Nxy)} of the Nei and Li was applied (Nei *et al*., 1979). where Nx= no. for alleles in the X genotype and Ny= no. alleles for the Y genotype and Nxy= no. of common alleles in both genotypes and Dxy= dissimilarity distance between the two genotypes. Genetic distance data were used to develop a phylogenetic tree and estimate clonal relationships among the isolates based on similarity coefficients ≥ 85%. For this purpose, the Molecular Evolutionary Genetics Analysis software (MEGA11) and POPGENE-32 software were used (Waters *et al*., 2012).

RESULTS

Figure 1 illustrates the band patterns produced by the ERIC-PCR. These bands represent enterobacterial repetitive intergenic consensus (ERIC) repeats whose copy numbers vary among different isolates. This variation provides the basis for estimation of genetic diversity among the isolates. A total of 24 bands were generated that ranged in size from 100 bp to 3000 bp. Only clearly visible and reproducible bands were considered for genetic distances estimations. The maximum number of bands observed was seven in three isolates, whereas the minimum was one, observed in ten strains. The phylogenetic tree for the *E. coli* isolates is shown in Figure 2. All the two hundred isolates were grouped into 98 clusters based on a similarity coefficient of ≥ 85%. Each clusters had two isolates, except C56, C40 which had three isolates and C63 which had four isolates. Clonal relationships according to location are summarized in Table I. Generally, isolates from the same location or nearby locations were genetically more similar. In contrast, isolates from geographically distant locations were genetically less related. The findings represent the clonal spread of *E. coli* in milk.

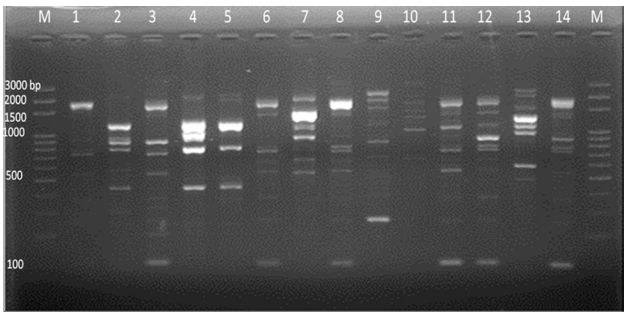

Fig. 1. ERIC-PCR products. Lane M = DNA ladder, Lane 1-14= *E. coli* samples.

Table I. Location-based distribution of *E. coli* clusters.

Location of sample collected	Clusters	*E. coli* isolates
Shaheen camp, Tehkal, Arbab Road, Abdra road and University town	C78	1, 2
	C79	3, 4
	C64	5, 6
	C81	7, 8
	C82	9, 10
	C70	11, 12
	C66	13, 14
	C67	15, 16
	C74	17, 18
	C75	19, 20
	C71	21, 22
	C73	23, 24
	C11	25, 26
	C12	27, 28
Board Bazar, Danishabad, Nemat Mahal and Tajabad	C76	41, 42
	C77	43, 44
	C5	45, 46
	C43	47, 48
	C44	49, 50
	C10	51, 52
	C46	53, 54
Saddar and Nouthia	C95	29, 30
	C93	31, 32
	C94	33, 34
	C97	35, 36
	C98	37, 38
	C96	39, 40
Gulberg 1, 2 and 3	C47	55, 56
	C45	57, 58
	C80	59, 60
	C83	61, 62
	C84	63, 64
	C85	65, 66
Ring Road	C87	67, 68
	C88	69, 70
	C89	71, 72
	C90	73, 74
	C86	75, 76
Nasir Bagh Road (Police Colony, Wazir Bagh, Regi, Askari Colony, Malkhander)	C49	101, 102
	C38	103, 104
	C32	105, 106

Table continues on next page.....

Location of sample collected	Clusters	Isolates name
	C33	107, 108
	C36	109, 110
	C34	111, 112
	C35	113, 114
Kohat Road, Hazar Khwani, Jameel Chowk	C22	77, 78
	C23	79, 80
	C24	81, 82
	C37	83, 84
	C13	85, 86
	C14	87, 88
Karkhano Market	C50	89, 90
	C51	91, 92
	C39	93, 94
Chamkani	C52	95, 96
	C53	97, 98
	C48	99, 100
Hayat Abad	C63	191, 192, 197, 198
	C62	193, 194
	C61	195, 196
	C65	199, 200
Ashraf Road, Nishtarabad and Firdaus	C1	138, 139
	C18	134, 135
	C19	136, 137
	C1	140, 141
	C2	142, 143
	C25	144, 145
	C26	146, 147
	C3	148, 149
Forest Bazar and Palosi	C56	127, 128, 129
	C55	130, 131
	C54	132, 133
Warsak Road, Shahi baala	C91	115, 116
	C92	117, 118
	C6	119, 120
	C7	121, 122
	C8	123, 124
	C9	125, 126
Badbair	C30	181, 182
	C31	183, 184
	C27	185, 186
	C28	187, 188
	C60	189, 190
Qisa Khwani, Ghanta Ghar, Khyber Bazar	C68	161, 162
	C69	163, 164
	C15	165, 166
	C16	167, 168
	C58	169, 170
Dalazak road	C59	171, 172
	C57	173, 174
	C20	175, 176
	C21	177, 178
	C29	179, 180
Gulbahar	C4	150, 151
	C41	152, 153
	C42	154, 155
	C40	157, 158, 156
	C72	159, 160

Table continues on next column.....

DISCUSSION

ERIC-PCR has been utilized to assess genetic diversity of *E. coli* isolated from various sources. In the present study, 200 *E. coli* isolates from 300 milk samples collected from shops across Peshawar City were analyzed using ERIC-PCR. A total of 98 clusters were identified. High genetic similarity was observed among isolates collected from same areas, while high genetic diversity was observed among isolates from different areas. Samples collected from interconnected and proximate areas were grouped in the same clusters, and cluster C64 contained two isolates from different but adjacent areas, namely Tehkal and Shaheen camps. These two areas are in proximity and the milk supply to Shaheen camp is predominantly from vendors in the Tehkal area. This suggests the clonal spread of *E. coli* in these areas. Similar observations were made in other areas. The samples collected across the Nasir Bagh Road were clustered together. These results indicate that the supply of milk across these areas could be identical. The conditions of the shops, handling procedures, and supply chain processes seem to share notable similarities. Cluster C63 comprises four strains, isolated from samples collected from Hayatabad. Cluster C56 contained three isolates from different but adjacent areas. It was observed that samples from geographically distant areas did not cluster together indicating the circulation of diverse strains in the region. These findings suggest a multiple source contamination of milk samples across the region and clonal dissemination within adjacent areas.

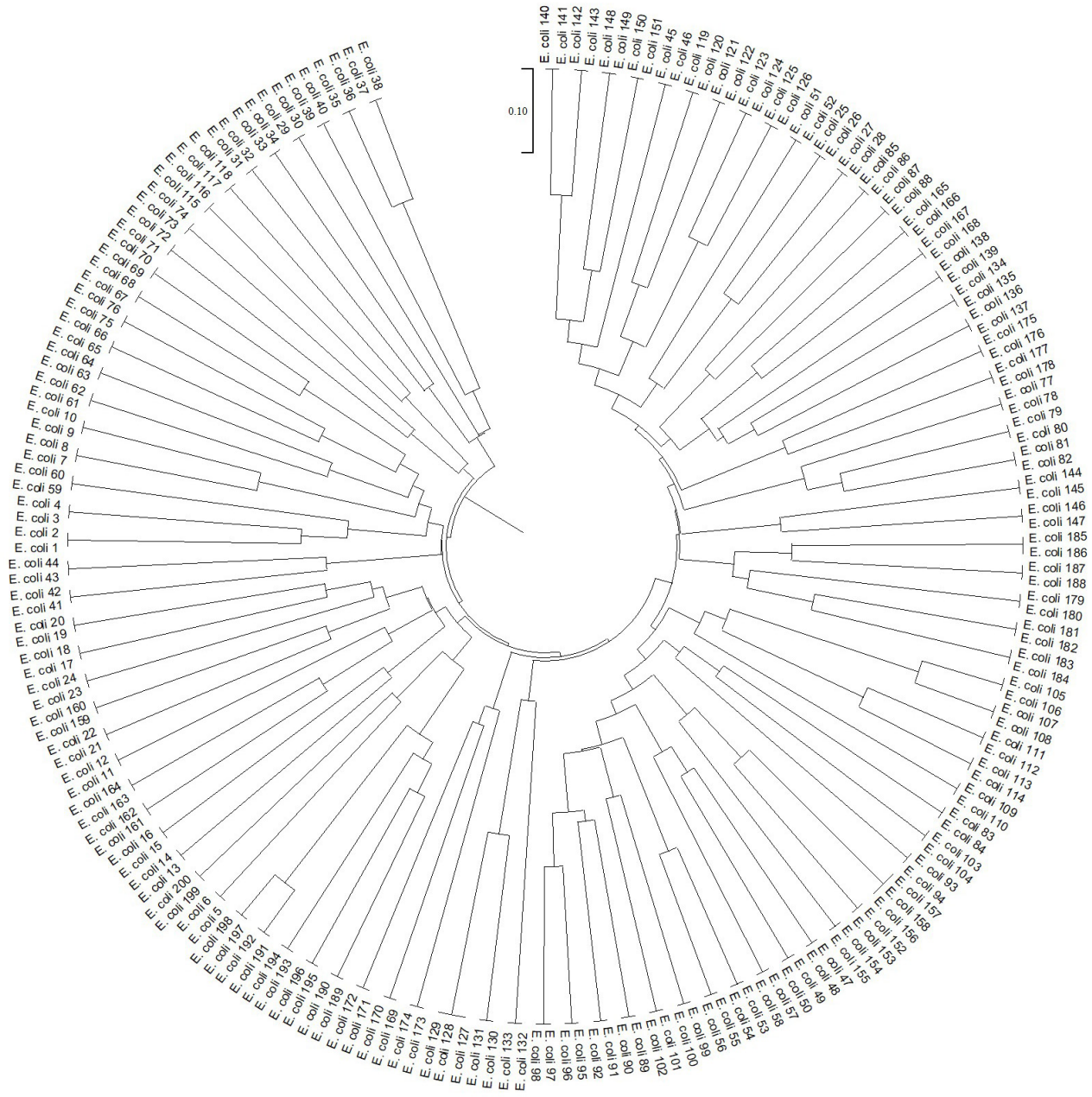

Fig. 2. Phylogenetic tree: The tree was generated using the MEGA 11 software. The isolates were clustered into groups based on genetic similarities.

The results of this study are consistent with the findings of several other studies. A study on pasteurized milk samples reported 90 clusters of *E. coli* with one clone circulating in multiple locations (Oltramari *et al*., 2014). This study also reported clusters that contained *E. coli* isolated from different areas suggesting common contamination sources and failure of the pasteurization process. Hoffmann *et al*. (2014) reported high genotype variability among eighty-seven *E. coli* isolates from pasteurized milk indicating the potential application of ERIC in epidemiological studies. They speculated the existence of multiple routes of milk contamination leading to high genetic diversity. In contrast to the findings of this study, a study from Egypt indicated the absence of endemic strains as high genetic diversity was observed among *E. coli* isolated from dairy farms including milk

samples (Awadallah *et al*., 2016).

Apart from milk samples, ERIC-PCR has also been utilized to assess genetic diversity among *E. coli* isolates from various food, environmental, and clinical samples. Yar *et al*. (2022) employed this method to determine genetic diversity among *E. coli* isolates from animals (milk, feces), environment (waste water, soil), and human. They observed high genetic diversity among isolates from the environment. The results also revealed that strains from different sources were grouped into single clusters indicating the possible dissemination of these strains from animals and the environment to human and vice versa. A high genetic heterogeneity among *E. coli* isolates of fish origin was reported in another study that was attributed to multiple strains circulating in the area (Alttai *et al*., 2023). An ERIC-PCR analysis of *E. coli* from frozen shrimp, beef and human showed similarities among the isolates from these sources and it was speculated that the food *E. coli* could act as human pathogens based on common ancestry (Alsultan and Alhadi, 2022). Several studies have also demonstrated the usefulness of ERIC-PCR for estimating genotype variability among bacterial strains from other sources (Ying *et al*., 2015). ERIC-PCR has been extensively utilized in clinical settings to control and prevent the spread of infections, identify the source of infection, and develop strategies for improved disease management. This method has been employed for numerous pathogenic bacteria in clinical samples i.e. *Klebsiella, E. coli, Salmonella* spp. *Acinetobacter baumannii* etc (Secundo de Souza *et al*., 2015; Jena *et al*., 2017; Aljindan *et al*., 2018; Sedighi *et al*., 2020; Movahedi *et al*., 2021).

The findings of the study provides insights into the genetic diversity, clonal relatedness and patterns of dissemination of milk contaminating *E. coli* isolates in the region. High genetic diversity among isolates form different areas shows the high adaptability nature of these isolates that could also contribute towards possession of diverse virulence factors, antimicrobial resistance mechanisms and other characteristics for flourishing in diverse environments. Whole genome sequencing studies could further elaborate these characteristic of the isolates. The findings are important with respect to public health as *E. coli* are known to cause various infections in human and their spread through milk highlight the need for proper hygienic practices during all stages of milk supply to the consumers. For the safety of consumers such studies should be routinely carried out to monitor the presence of bacterial contaminants in milk.

CONCLUSION

ERIC-PCR was found to be useful in estimating the genetic heterogeneity among *E. coli* isolates recovered from milk that could supplement routine epidemiological studies. The results indicate that many strains of milk contaminating *E.coli* are circulating in the region. These could be pathogenic in nature posing serious threat to human. High genetic diversity of *E. coli* isolates points to a multi-source contamination of milk along the supply chain. In order to guarantee milk safety, deliberate steps should be taken to enforce hygienic practices during milk handling from production to the consumer.

DECLARATIONS

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Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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