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Research Article

Molecular Detection and Characterization of Virulence Genes in Escherichia coli Isolated from Drinking Water and Raw Food Sources

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Abstract

Poor hygiene results in elevated levels of bacterial contamination in drinking water and raw food. *Escherichia coli* (*E. coli*) is one good indicator that shows the level of contamination in community water supply. *E. coli* harbor pathogenic and antibiotic resistance genes that can result in morbidity. The aim of the study is to assess the pathogenicity and antibiotic resistance prevailing in water and food samples collected from Kohat. Twenty samples were collected and subjected to various tests, including biochemical assays and polymerase chain reaction (PCR) to identify *E. coli*. Antibiogram assays were performed using Kirby disc diffusion method. PCR was employed to identify virulence genes Shiga toxin 1 and 2 (*Stx1* and *Stx2*). 70% of samples were found to have bacteria in them, and the most common were *E. coli* and other *Enterobacteriaceae*. Additional testing of *E. coli*-positive samples confirmed positive Triple Sugar Iron (TSI) results but negative citrate and urease tests. Antibiotic susceptibility testing revealed that most of the bacteria strains are resistant to penicillin, ampicillin, ceftazidime, and cefixime, while some samples showed susceptibility to amoxicillin plus clavulanic acid, cefoxitin, cefepime, cefoperazone + sulbactam, levofloxacin, ceftriaxone, ciprofloxacin, clarithromycin, azithromycin, and doxycycline as per Clinical and Laboratory Standards Institute (CLSI) standards. Three of the isolates were subjected to PCR for the detection of *STX1* and *STX2* genes confirming the presence of the *Stx2* gene in the two *E. coli* isolates, while *Stx1* was absent. Sequence analysis of *Stx2* gene showed >98% identity with GenBank sequences. Finally, our comprehensive study confirmed that *E. coli* is harmful in raw food and drinking water. Phylogenetic analysis, virulence factor discovery, antibiotic resistance profiling, and more help us identify *E. coli* contamination. This highlights the need for cleanliness and wastewater management.

Keywords: Antibiotic, *Escherichia coli*, raw food, virulence, antibiotic assay

1. Introduction

The coliform bacteria *E. coli* is a facultative anaerobe that is gram-negative and rod-shaped. In humans and other mammals, *E. coli* is a typical component of the normal flora of the lower intestine (Singleton 2004). The majority of *E. coli* strains are known as human pathogens, but some serotypes like *Enteropathogenic E. coli* (*EPEC*) and *Enterotoxigenic E. coli* (*ETEC*) are dangerous. They are the source of food contamination as well as severe food poisoning. The majority of *E. coli* strains are parts of the normal gut microbiota and do not pose a threat to humans, in fact, they may even be helpful since they produce vitamin K and prevent dangerous bacteria from occupying the intestine. It has been observed that 0.1 percent of the gut microbiota is established by facultative anaerobes like *E. coli* (Eckburg et al. 2005). The primary method by which pathogenic strains spread disease is through fecal-oral transmission. Because these cells may live outside the body for a brief period of time, they are useful indicator organisms to look for fecal contamination in environmental samples (Feng et al. 2002).

Infection with pathogenic *E. coli* commonly results in the manifestation of severe diarrhea. Globally, the annual incidence of diarrheal illnesses amounts to over 1.7 billion. Diarrheal illness is the second leading cause of mortality among children aged 5 and under. It can be effectively prevented by the implementation of improved environmental sanitation practices, while its treatment can be facilitated through the administration of appropriate antibiotics. However, it has been observed that specific strains of bacteria develop resistance to antibiotics over time (Collignon 2009, Tadesse et al. 2012). The occurrence of pathogenic *E. coli* is attributable to soil and water contamination from animal excrement. The issue of unsafe food poses significant economic threats, especially in a globalized context (Mcauley et al. 2014).

Enterohemorrhagic *Escherichia coli* (EHEC) is a notable pathogen that has garnered worldwide focus due to its connection with numerous significant foodborne outbreaks. EHEC leads to both non-bloody and bloody diarrhea, as well as hemolytic uremic syndrome (HUS), affecting individuals across different age groups. It is important to acknowledge that people in both younger and older age groups exhibit increased vulnerability to these illnesses. The notable serotype of *E. coli* associated with EHEC is O157:H7, which has been implicated in numerous illness outbreaks across North America, Europe, and Japan (Meng et al. 2012).

The Shiga toxins (*stx1* and *stx2*) genes play a crucial role in the synthesis of Shiga toxins, which are associated with diarrhea, hemorrhagic colitis, and HUS. *Stx1* and *Stx2* are crucial in the pathogenesis of gastroenteritis, hemorrhagic colitis, and HUS (Friedrich et al. 2007). Bacteriophages demonstrate significant mobility and contain genes that control *Stx* expression, facilitating horizontal gene transfer and resulting in the development of new *Stx* variations or infections (Sylvia, Helge, and Herbert 2004). Multiple variants of the *stx1* and *stx2* genes have been found (Lee et al. 2007), the prototype and *stx2c* are particularly noteworthy because of their stronger association with HUS compared to *stx1* and *stx2* activatable (Lee et al. 2007). The dynamics of *stx*-phages contribute to the instability of *stx* genes in both in vivo and in vitro environments, resulting in their loss during subculturing and infection.

Pathogens can originate from contaminants present in the surrounding environment, such as soil and water, as well as from animals and humans. The majority of cases in the foodborne *E. coli* pandemic were attributed to the consumption of raw and contaminated food, including ground beef hamburgers and undercooked vegetables (Park et al. 2014). Outbreaks were attributed to food prepared by restaurants and catering facilities, with the fear that infected food workers may have been the source of contamination (Garitano et al. 2011). The most recent and serious outbreak occurred in Germany in 2011 as a result of STEC 104: H4, which led to 3816 confirmed Shiga toxin-producing *E. coli* (STEC) infections and 54 fatalities, 32 of which were related to HUS. It was determined that HUS primarily affects youngsters. Nonetheless, 852 cases of HUS, or 89% of the 3816 STEC infections occurred, accounting for 22% of the total. The source of the infection was found to be raw sprouts (Frank et al. 2011). The aim of the study is to assess the pathogenicity and antibiotic resistance prevailing in water and food samples collected from Kohat. The current study includes the collection of raw food samples from numerous sources, which were then analyzed in the microbiology laboratory to isolate and identify bacterial strains. Biochemical, molecular, and antibiotic susceptibility analyses were conducted to characterize these isolates. Phylogenetic analysis was performed to determine the evolutionary relationships among the bacterial strains.

2. Materials and Methods

2.1. Collection of food samples

Food samples were systematically gathered from various regions within the local marketplace of Kohat city, located in the Khyber Pakhtunkhwa province of Pakistan. Various varieties of raw meat samples, including beef, fish, poultry, and uncooked meals, have been collected. The specimens were gathered in aseptic containers and conveyed to the microbiological laboratory at the Department of Pharmacy, Kohat University of Science and Technology (KUST) within a 24-hour timeframe, following the established protocol (Willey, Sherwood, and Woolverton 2011). All of the gathered specimens were appropriately labeled and processed within the same day.

2.2. Bacterial isolation and identification using raw foods

Each specimen (1 gm) was introduced into 10 ml of normal saline solution. The mixture was incubated for 1.5 hours in a shaker incubator. Following the incubation period, a volume of 100 to 200 µl of saline solution was extracted from the sterile container and subsequently applied to MacConkey media. The solution was evenly distributed across the media surface using a sterile spreader, and the resulting plate was accurately labeled. Subsequently, the plate was placed in the incubator, which was kept at a stable temperature of 37°C, and allowed to incubate for a period of 24 hours (Zhang et al. 2021). Various characteristics of the colony, such as its size, color, shape, margin, and opacity, were meticulously examined (Garrett et al. 2019).

2.3. Biochemical Analysis

Biochemical assays were conducted to identify bacterial isolates, concentrating on the enzymatic activities linked to the degradation of substances such as urease, citrate, TSI, and Gram staining. All of these tests were carried out in accordance with established protocols (Hua et al. 2022, Urgessa et al. 2023, Liu et al. 2019, Webber, Wallace, and Burnham 2022, Widodo et al. 2022).

2.4. Antibiotic susceptibility test

Susceptibility testing was performed to determine the antibiotic capable of inhibiting the growth of bacteria associated with a specific condition. In order to reach the desired goal, different antibiotic discs were applied to plates with Muller Hinton Agar (MHA) that had been inoculated with the isolates for a duration of 24 hours (Morales-García and Pacheco-Delgado 2021). The plates were examined for cefoxitin, ciprofloxacin, levofloxacin, doxycycline, amoxicillin, cefepime, clarithromycin, azithromycin, ceftriaxone, ampicillin, cefixime, ceftazidime, cefoperazone, and penicillin. The scale was used to measure the zone of inhibition (Saccà and Lodesani 2020) according to CLSI recommendations. After the incubation period, the diameter of the zone was measured and the outcomes were interpreted as resistant, intermediate, or vulnerable based on established protocols. The measurement of the zone of inhibition provides valuable insights into the level of resistance exhibited by microorganisms, and it also offers important indications regarding the presence of resistance genes (Webber, Wallace, and Burnham 2022).

2.5. Molecular characterization

Using the phenol-chloroform technique, DNA was extracted (Hoffman 1997). Following centrifugation, the pellet was dissolved in 567 µl of T.E. buffer, 3µl of 20 mg/ml proteinase K, and 30ul of 10% SDS, and it was then incubated for an hour at 37 °C. The supernatant was then removed. In the next step, 100 µl of 5M NaCl was added and mixed thoroughly. Then 80 µl of CTAB/NaCl solution was introduced and incubated at 65 °C for 10 minutes.

After this step, 0.7 ml of chloroform, and isoamyl alcohol were added to the contents of the Eppendorf tubes and were centrifuged for 5 minutes at 12,000 rpm. After centrifugation, the

Table 1. Specific primers were used for amplification of stx1 and stx2 genes.

Primer sequence	Size	Reference
Stx1 F CTT CGG TAT CCT ATT CCC GG **Stx1 R GGA TGC ATC TCT GGT CAT TG**	**484**	(Tahamtan, Hayati, and Namavari 2010)
Stx2 F CCA TGA CAA CGG ACA GCA GTT **Stx2 R CCT GTC AAC TGA GCA GCA CTT TG**	**779**	

supernatant was moved to a new tube, and equal amounts of phenol, isoamyl, and chloroform were added and centrifuged. The supernatants were transferred to a different tube for precipitation and centrifuged for 5 minutes. After transferring the supernatants to a new tube, 0.6 volume of isopropanol was added for precipitation. DNA was centrifuged for five minutes in 70% ethanol to remove any remaining material. After letting the pallet air dry in an Eppendorf tube at room temperature, it was resuspended in 100 µl of TE buffer. The extracted DNA was examined using 1% agarose gel electrophoresis before being preserved for further use at minus 4°C (Köchl, Niederstätter, and Parson 2005).

2.6. Polymerase Chain Reaction (PCR)

The DNA in the samples was amplified by Polymerase chain reaction (PCR). A total of 25 µl reaction mixture utilized in PCR consisted of the following components: 12 µl of master mix, 1 µl of forward primers, 1 µl of reverse primers, 3 µl of template DNA, and 8 µl of water for each of the *Stx* specific primers (Table 1). The PCR process entails amplifying DNA via a sequence of 30 cycles. The amplification conditions comprised denaturation at 94 °C for 30 seconds, primer annealing at 56 °C for 30 seconds, and primer extension at 72 °C for 30 seconds. The PCR tubes were incubated at a temperature of 72°C for a duration of 10 minutes. The PCR results were maintained at a temperature of -4 °C and subsequently analyzed using 2% agarose gel electrophoresis. The amplified PCR results were then sequenced and analyzed to determine the phylogenetic relationship among the bacterial isolates and their nearest lineages. The analysis was performed utilizing the NCBI BLAST online bioinformatics tool (Tahamtan, Hayati, and Namavari 2010).

2.7. Gel electrophoresis

A 1.5% agarose gel was prepared utilizing 1X Tris-Borate-EDTA (TBE) buffer for the purpose of electrophoresis. The agarose was fully dissolved by heating. A concentration of 0.5µg/ml of ethidium bromide was introduced into the system under conditions that ensured viability. The combs were meticulously positioned within the gel tray, followed by the pouring of the gel, which was then permitted to solidify. Once the gel solidified, the combs were meticulously removed from the tray, and the gel was positioned into a tank that maintained a continuous flow of 1XTBE buffer. The amplified PCR product, along with the loading dye, was meticulously loaded into a well of a 1.5% agarose gel using a micropipette. The total volume of the PCR product along with the loading dye amounted to 2 µl. Additionally, a volume of 5µl of ladder was introduced into one of the wells. The gel electrophoresis procedure was carried out, and upon completion, the gel was placed under a trans-illuminator to capture images and record measurements (Takemori et al. 2022).

2.8. Sequencing of the purified PCR product

After purification, the PCR product was mixed with 5µl of forward and reverse primers and then sent to Macrogen for sequencing analysis. The nucleotide sequence of each isolate underwent analysis with the Blastn tool to identify matches with sequences in the gene bank database. The

Table 2. Biochemical analysis of bacterial isolates.

S. No	Citrate Utilization Test	Urease Test	Triple Sugar Iron Agar Test				Remarks
			Slope	Butt	H_2S	Gas	
Sample 1	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 2	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 3	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 4	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 5	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 6	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 7	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 8	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 9	-ve	-ve	R	Y	+ve Weak	-ve	*S. typhi*
Sample 10	-ve	-ve	R	Y	-ve	+ve	*S. paratyphi-A*
Sample 11	-ve	-ve	Y	Y	-ve	+ve	*E. coli*
Sample 12	-ve	-ve	R	Y	+ve Weak	-ve	*S. typhi*
Sample 13	-ve	+ve	Y	Y	-ve	+ve	*E. coli*
Sample 14	-ve	-ve	Y	Y	-ve	+ve	*E. coli*

Key, Y=yellow, R=red, H_2S= Hydrogen sulphide (blackening).

alignment with the lowest expected value (e-value), indicating the probability of non-random alignments, was chosen to optimize the volume of blast production.

2.9. Nucleotide BLAST analysis from NCBI

The gene sequences of *Stx1* and *Stx2* from our isolates were deposited in the NCBI nucleotide database for identification. The sequences that exhibited the highest similarity to our isolates, as determined by percent identity and query coverage, were selected. These selected sequences were then aligned using the MUSCLE program, employing multiple sequence alignments.

2.10. Phylogenetic tree construction

To determine the phylogenetic relationships between the bacterial isolates and their closest lineages, a multiple sequence alignment was conducted utilizing the MUSCLE tool within the MAGAX software. This process of alignment entailed the comparison of the isolates with sequences that exhibited the highest degree of similarity. The Maximum Likelihood approach in MEGAX was employed to create phylogenetic trees for each isolate. Before constructing the phylogenetic tree, the alignment of all similar sequences to their respective isolates was conducted using the MUSCLE tool in MEGAX software (Yang, Wang, and Qian 2016).

3. Results

3.1. Bacterial Characterization

Samples were appropriately labeled with the name, date, and location. Many different colonies were found on the MacConkey Agar after 24 hours of incubation. We selected the Lactose fermenter bacterial colonies. The Lactose fermenter colonies were sub-cultured on fresh

Table 2: Susceptibility data of the isolates

Antimicrobial agents	Codes	Zone Diameter/ CLSI standards			Zone of Inhibition (mm)													
		S	I	R	Sp1	Sp2	Sp3	Sp4	Sp5	Sp6	Sp7	Sp8	Sp9	Sp10	Sp11	Sp12	Sp13	Sp14
Penicillin G	P	≥ 17	14-16	≤ 13	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amoxicillin + Clavulanic acid	AMC	≥ 18	14-17	≤ 13	0	0	0	0	23	0	0	0	0	0	0	0	0	0
Ampicillin	AMP	≥ 18	14-17	≤ 13	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cefoxitin	FOX	≥ 18	15-17	≤ 14	24	19	20	0	0	20	30	29	0	14	0	0	0	15
Cefepime	CPM	≥ 25	-	≤ 18	0	12	16	16	10	15	27	0	18	16	19	15	0	0
Cefoperazone +Sulbactam	CES	≥ 21	16-20	≤ 15	23	28	22	26	0	22	33	0	20	20	22	19	19	17
Ceftriaxone	CRO	≥ 23	20-22	≤ 19	0	25	21	24	20	22	29	0	20	18	21	19	14	0
Ceftazidime	CAZ	≥ 21	18-20	≤ 17	0	0	0	0	0	10	0	0	0	0	0	0	0	0
Cefixime	CFM	≥ 19	16-18	≤ 15	0	0	0	0	0	18	0	0	0	0	0	0	0	0
Levofloxacin	LEV	≥ 17	14-16	≤ 13	32	30	22	26	21	18	36	0	21	16	21	20	17	16
Ciprofloxacin	CIP	≥ 21	16-20	≤ 15	32	33	26	28	16	23	34	30	30	18	20	11	19	15
Clarithromycin	CLR	≥ 17	-	≤ 11	0	0	20	0	26	0	14	0	0	0	11	0	16	10
Azithromycin	AZM	≥ 13	-	≤ 12	18	0	20	20	27	0	24	34	0	0	14	10	16	13
Doxycycline	DO	≥ 14	11-13	≤ 10	0	11	14	0	28	16	24	0	0	0	0	0	0	0

MacConkey Agar media to isolate pure culture. Among these 14 positive lactose fermenter Enterobacteriaceae species were recognized; 11 samples of *E. coli*, 2 were *Salmonella typhi*, and 1 as *Salmonella paratyphi A* using Gram staining technique, urease, citrate, and TSI test. *E. coli* is a lactose fermenter bacterium that turns into pink colonies

Bacterial isolates were identified and described by biochemical techniques, including citrate utilization, urease assay, and triple sugar iron agar test. The recognized species of isolates, based on biochemical analysis, were *E. coli, S. Typhi, and S. Paratyphi A*. The results of biochemical tests are shown in Figure 3 and Table 2.

3.2. Antibiotic susceptibility

All bacteria are resistant to penicillin, amoxicillin plus clavulanic acid, ampicillin, ceftazidime, and cefixime, except Sp5 in the case of amoxicillin plus clavulanic acid and SP6 in the case of Cefixime. Isolates found that bacteria were resistant to Ceftoxitin while sp4, sp5, sp9, sp10, sp11, sp12, and sp13 were found susceptible. Except sp7 and sp11 all isolates showed resistance against Cefepime. In the case of Cefoperazone + sulbactam and levofloxacin, all samples were found susceptible except Sp5 and Sp8, which were resistant to Cefoperazone + sulbactam and Sp8 to levofloxacin respectively. In the case of ceftriaxone, Sp1, 8, 10, 13, 14 were found resistant. Sp12 was found resistant against ciprofloxacin while in the case of clarithromycin, all strains were resistant except Sp3, 5, 7, and 13. Moreover, Azithromycin resistance was found in Sp2, 6, 9, 10, and 12 while in the case of Doxycycline, all samples were resistant except Sp2, 3, 5, and 7 as per CLSI standards (Table 3 and Figure 1).

3.3. Molecular Characterization

3.3.1. Isolation of genomic DNA from Bacterial isolates

Plates were streaked with a single colony and incubated overnight at 37 °C to extract DNA from bacterial isolates.

3.3.2. Amplification of *stx1* and *stx2* virulent genes

The Shiga toxin-producing virulence genes *stx1* and *stx2* were amplified from three bacterial

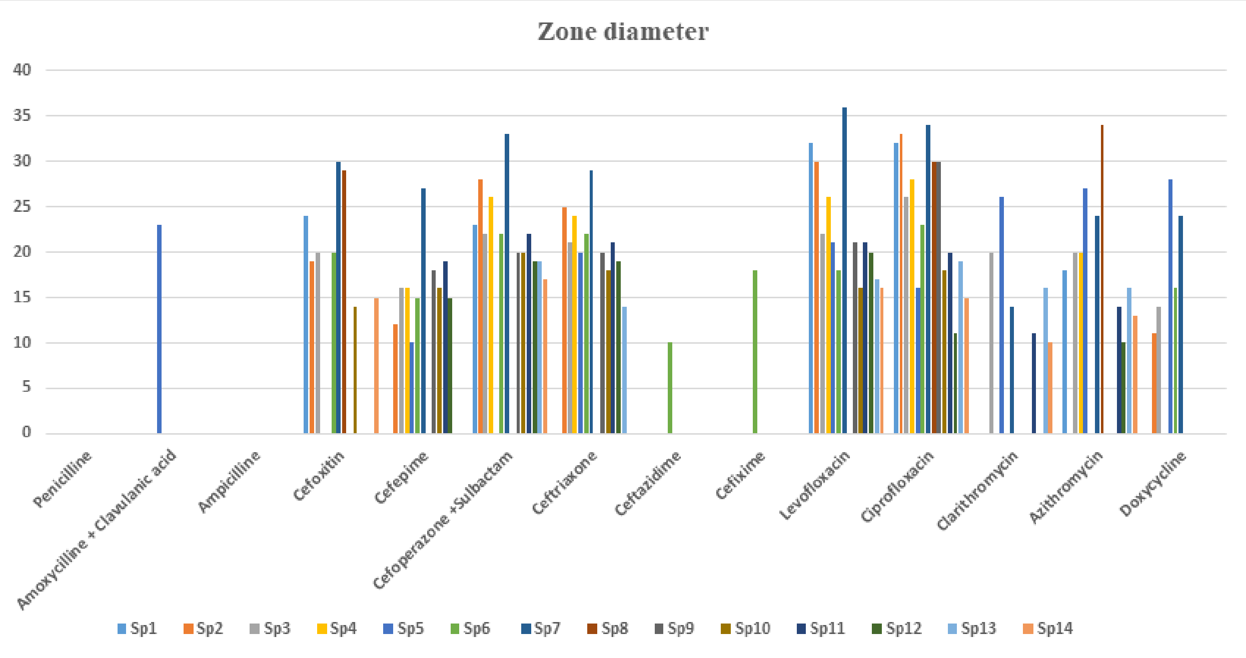


Figure 1: Graphical representation of Enterobacteriaceae isolates against antibiotics disc showing zone of inhibition.

isolate samples using previously described primer sets by PCR.

3.3.3. Sequence Analysis

The positive amplified products of PCR, obtained from sample 3 and sample 13 of *E. coli*, were sequenced commercially from Macrogen Company Korea. Sequences were trimmed and blasted using NCBI to check the similarity with the available sequences in the databases. The sequence of *Stx2* gene from sample 3 was determined to be 737 bp approximately. Analysis of sequence showed more than 99 % sequence similarities with *stx2* gene from Enterohemorrhagic *E. coli* available in the GenBank with accession numbers CP129383; and CP129345, followed by accession numbers, CP129263 respectively. Similarly, the sequence from sample 13 was determined to be 742bp approximately. Analysis of sequence shows maximum nucleotide sequence identity with available sequences in the GenBank, elucidating to be *stx2* gene of *E. coli*. The maximum identity of the sequence was determined 99 % with bacterial isolates accession numbers CP025840, LR134236, and CP061239, respectively Figure 4.

3.3.4. Phylogenic Analysis of Bacterial Isolates

MUSCLE incorporated in MEGA11 software and the percentage of replication tree in which the taxa clustered in bootstrap value (1000 repetitions) produced a phylogenetic dendrogram. Based on GenBank sequences with the highest nucleotide similarity, a phylogenetic tree of Shiga toxin-producing *E. coli* virulence gene *stx2* (from samples 3 and 13) was compared to previously described sequences.

Using the maximum likelihood feature included in the MEGA11 program and the alignments of certain *stx2* virulence genes from GenBank, a phylogenetic dendrogram was created, as seen in Figure 5. The tree shows that strong bootstrapping values were used to support each group in the dendrogram. Sample 3 showed that segregation with a bootstrap value (76%) with United Kingdom (CP09929.1) isolates and lies at the basal to the rest of the isolates, consistent with the idea that the identified isolates in this study belong to the same species. Our isolates were found to be very closely related, with extremely short branch lengths, to a reported bacterial isolate from the USA (CP070103.1).

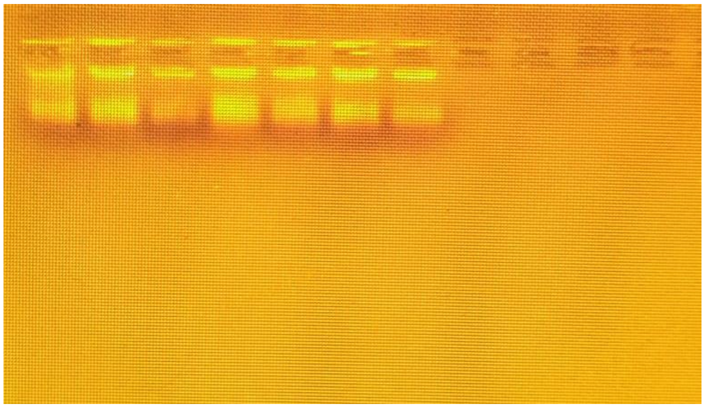


Figure 2: Isolation of genomic DNA from Bacterial isolates

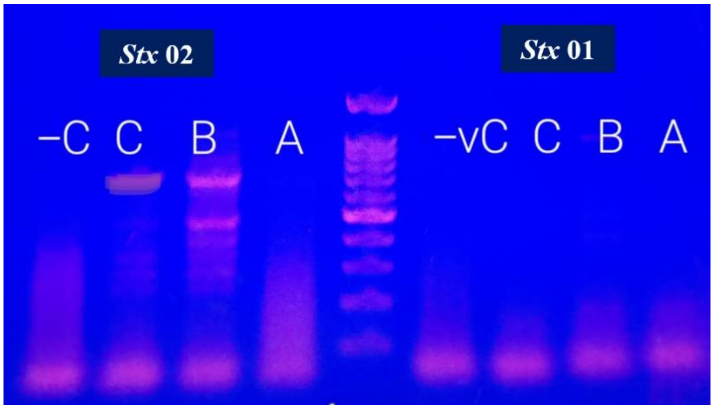
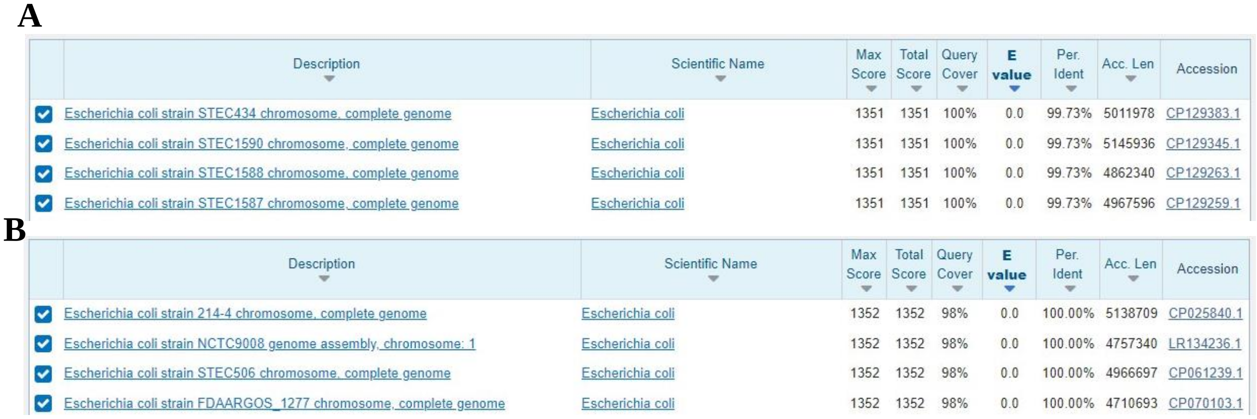


Figure 3: Gel electrophoreses of PCR amplicons from sample 1, sample 3 and 13, using primer sets of stx-1 and stx-2. Lane A is sample 1, Lane B is 3, Lane C is sample 13, C- negative control and Ladder.

4. Discussion

This study provides important insights into the characterization, antibiotic resistance, and genetic profiling of Enterobacteriaceae isolates obtained from raw food, drinking water, and milkshake samples collected in Kohat. A report from the Global Antimicrobial Resistance and Use System indicates that *E. coli* is the most encountered clinical organism globally (Torres et al. 2018). In addition to *E. coli,* we also identified other members of the Enterobacteriaceae family, including *S. typhi* and *S. paratyphi* A, however, our primary emphasis was on *E. coli.*

The identification of *E. coli, Salmonella typhi*, and *Salmonella paratyphi A* among lactose-fermenting bacterial colonies aligns with earlier research that has documented these pathogens in food and water settings. Targeted media, like MacConkey agar, combined with Gram staining and biochemical tests, successfully isolated and identified these species. The commonality of *E. coli* as a lactose fermenter corresponds with its notable



	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Escherichia coli strain STEC434 chromosome, complete genome	Escherichia coli	1351	1351	100%	0.0	99.73%	5011978	CP129383.1
☑	Escherichia coli strain STEC1590 chromosome, complete genome	Escherichia coli	1351	1351	100%	0.0	99.73%	5145936	CP129345.1
☑	Escherichia coli strain STEC1588 chromosome, complete genome	Escherichia coli	1351	1351	100%	0.0	99.73%	4862340	CP129263.1
☑	Escherichia coli strain STEC1587 chromosome, complete genome	Escherichia coli	1351	1351	100%	0.0	99.73%	4967596	CP129259.1

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Escherichia coli strain 214-4 chromosome, complete genome	Escherichia coli	1352	1352	98%	0.0	100.00%	5138709	CP025840.1
☑	Escherichia coli strain NCTC9008 genome assembly, chromosome: 1	Escherichia coli	1352	1352	98%	0.0	100.00%	4757340	LR134236.1
☑	Escherichia coli strain STEC506 chromosome, complete genome	Escherichia coli	1352	1352	98%	0.0	100.00%	4966697	CP061239.1
☑	Escherichia coli strain FDAARGOS_1277 chromosome, complete genome	Escherichia coli	1352	1352	98%	0.0	100.00%	4710693	CP070103.1

Figure 4: (A)The sequence of sample no.13 isolates accession number CP025840, LR134236 and CP061239 and (B) sample no.3 with accession numbers CP129383; CP129345, , CP129263 respectively.

prevalence in contaminated water and food sources.

The results reveal a concerning level of resistance to antimicrobial agents in bacterial isolates. All isolates demonstrated complete resistance to beta-lactam antibiotics, including penicillin, amoxicillin-clavulanic acid, ampicillin, ceftazidime, and cefixime, except for Sp5, which displayed susceptibility to amoxicillin-clavulanic acid. While most isolates showed susceptibility to ciprofloxacin, cefoperazone-sulbactam, and levofloxacin, specific cases of resistance highlight the necessity for customized antibiotic therapies. The observed resistance corresponds with investigations conducted in India, where *E. coli* has demonstrated considerable antimicrobial resistance, exhibiting 57% resistance to beta-lactams (Ghosh et al. 2022). This trend underscores the vital significance of judicious antibiotic application and continuous evaluation for resistance. It is essential to implement strong measures, including strict guidelines on antibiotic usage and initiatives aimed at improving public health education, to tackle the growing issue of resistance. Isolates that were reported earlier exhibited significant resistance rates to ampicillin (88.4%), amoxicillin and clavulanic acid (74.4%), ceftriaxone (74.4%), norfloxacin (74.2%), and cefuroxime (72.2%) (Niranjan and Malini 2014). In-depth investigation into genetic resistance mechanisms and improved monitoring is essential to guide clinical practices and public health strategies.

The successful amplification and sequencing of *stx2* virulence genes from *E. coli* isolates (Samples 3 and 13) confirms the presence of Shiga toxin-producing strains. The significant sequence similarity (>99%) with recognized *stx2* genes in the GenBank database highlights the pathogenic potential of these isolates. This discovery prompts significant worries due to the considerable consequences for community health associated with *E. coli* strains containing *stx2*, which are related to critical conditions such as HUS. Shiga toxin production *Escherichia coli* (STEC) represents a varied collection of isolates encompassing multiple serogroups and serotypes. They all possess a viral element in their genomes that carries the genes responsible for Shiga toxin production (Melton-Celsa 2014). Certain

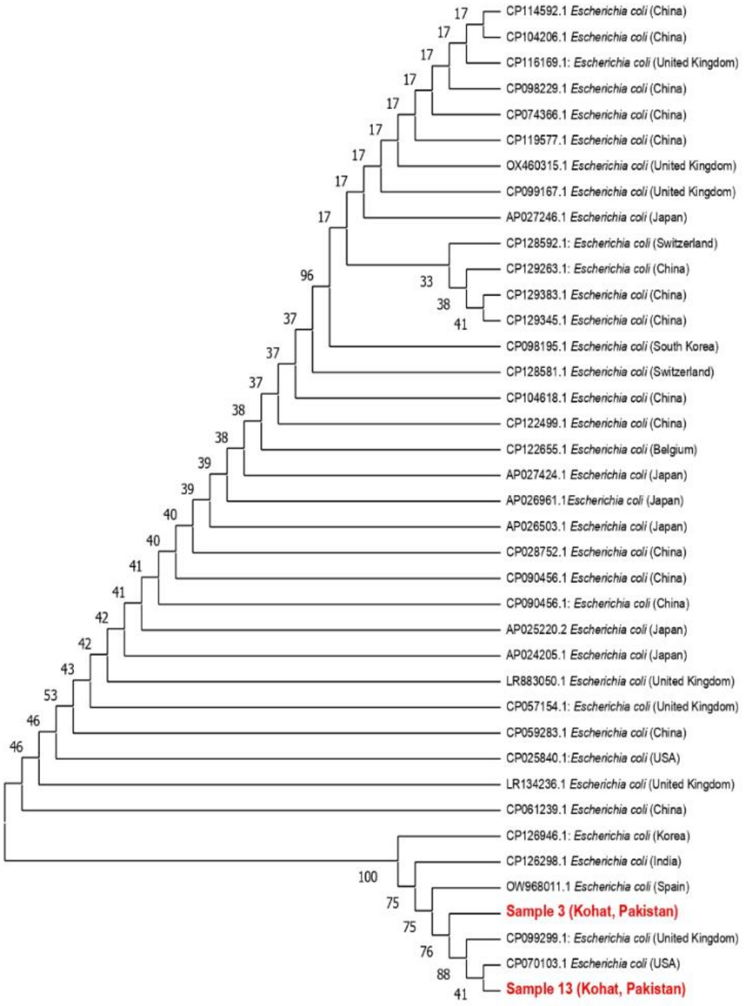


Figure 5: A Phylogenetic tree of Shiga toxin-producing *E. coli* virulence gene stx-2 (from sample 3 and 13).

serotypes, particularly the prevalent O157:H7, have emerged as significant microorganisms present in food and water, capable of causing various illnesses, including HUS and diarrhea (Zumbrun et al. 2013). Animals harbor STEC isolates, primarily with cattle serving as the principal source of these strains. They were also the origin of impurities in food and water. Cattle and various other animals host a diverse range of serotypes, yet only certain STEC serotypes, known as EHEC, have been identified as causing illness in humans (Torres et al. 2018). A study published in 2014 assessed the global prevalence of human STEC infections and related fatalities. The results revealed that STEC accounts for approximately 2.8 million cases of acute illnesses annually, leading to 3890 occurrences of HUS, 270 instances of end-stage renal disease, and 230 fatalities. The execution of health programs across various countries has resulted in a reduction in the frequency of outbreaks. Moreover, during an outbreak, the adverse effects faced by those affected have also been mitigated (Farfan and Torres, 2012). There have been numerous studies that show the existence of bacteriophages with *stx* genes in wastewater in addition to bacteria that carry the gene. For instance, phages containing the *stx2* gene, which can have concentrations of up to

1010 gene copies/mL of wastewater, were detected in 70% of urban sewage samples and 94% of animal wastewater samples (Muniesa, Jofre, and Microbiology 1998). A lower proportion of *s*-specific phages, detectable in 16%–19% of phage plaques and thought to occur at a frequency of 1–10 phages/mL of sewage water, contain the *stx* gene (Dumke et al. 2006). These investigations show that *stx*-containing bacteria and bacteriophages were widely distributed in wastewater. The chromosomal location of the *Stx* genes in *S. dysenteriae* has been identified (Blinova et al. 2023). Bacteriophages that encode *Stx1* and *Stx2* independently have been identified from different strains of EHEC. These phages have now become emblematic of the prophages that carry the genes responsible for producing *Stx* (Fagerlund et al. 2022). The *stx2* gene phylogenetic tree showed a strong relationship between our isolates and those from the UK and USA. This suggests trade or migration might spread linked *E. coli* strains in Kohat. Sample 3 association with UK isolates and central position in the dendrogram may indicate an evolutionary divergence or different lineage in the nearby environment. The results emphasize the necessity for strict food and water contamination prevention measures. The growth of antibiotic resistance and the discovery of virulent genes make bacterial infection management and transmission difficult. For community well-being, prioritize consistent food and water monitoring to identify small organisms. Epidemiological research should also include a genetic study of harmful strains to monitor and suppress outbreaks produced by powerful and resilient pathogens. This study is restricted by its area and subject matter, yet its results are significant. To increase relevance, future studies should cover a broader geographic range and larger sample numbers. Furthermore, complete isolated sequencing may reveal genetic factors that affect virulence and resistance.

5. Conclusion

The present study effectively detailed the identification of bacterial isolates from raw food, water, and milkshake samples, pinpointing *E. coli*, *S. Typhi*, and *S. Paratyphi A* using biochemical and molecular techniques. Testing for antibiotic susceptibility showed a significant level of resistance among isolates, underscoring the importance of careful antibiotic application. Analysis of *stx2* gene sequences revealed more than 99% similarity to reference strains in GenBank, highlighting the pathogenic potential of these isolates. Phylogenetic analysis revealed strong connections with isolates from the USA and UK, highlighting the worldwide importance of these discoveries. Future plans include monitoring antimicrobial resistance to guide treatment and avoid dissemination. We can better comprehend the genetic diversity and pathogenicity of these isolates by sequencing their complete genomes. However, preventing bacterial spread requires improving food and water safety. Whereas vaccines targeting antibiotic-resistant bacteria and environmental reservoir studies will improve control and minimize public health risks.

Competing Interests

The authors declare no competing interests.

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Study Approval

This study was approved by the Department of Pharmacy, Kohat University of Science & Technology, Kohat, Pakistan

Consent Forms

Not applicable

Data Availability

All the data related to this manuscript are available with the authors.

Author Contributions

Main idea and conceptualization, initial draft by FU, literature collection, and review by TAH, MNS & FU, graphics, language, and grammar by TAH &MNS, analysis and proofreading by FU, review editing by FU and final draft by TAH, MNS and FU. All authors read and approved of the final manuscript.

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