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Coliform Analysis and Molecular Characterization of *E. coli* in Krishna River Water near Vijayawada City

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Abstract

The primary aim of this study was to investigate the occurrence, antimicrobial resistance patterns, virulence gene profiles, and genetic diversity of *Escherichia coli* isolates obtained from river water samples collected across different locations along the Krishna River in Vijayawada, India. A total of 27 samples from 9 different ghat locations along the course of Krishna River (in triplicate) in the Vijayawada city landscape were collected and analysed for presumptive coliform tests. From the positive presumptive coliform test samples, 44 *E. coli* isolates were recovered and confirmed by biochemical tests and molecular tests targeting *16s rRNA*. All 44 *E. coli* isolates were further subjected for the detection of virulence genes i.e. stx_1, stx_2, *eaeA* and *hlyA* genes using multiplex PCR. Out of 44 isolates two isolates carried all four genes, one isolate harbored stx_2, *eaeA* and *hlyA* genes and one isolate showed the presence of *eae* and *hlyA* genes in combination. The 44 *E. coli* isolates were subjected to antimicrobial sensitivity/resistance against 12 antimicrobial agents by disc diffusion method. Higher resistance was observed for naladixic acid (40.9%) followed by ampicillin (38.63%), ceftazidime (34%) and ceftriaxone (27.27%). Out of 44 isolates of *E. coli*, ten isolates harboured β-lactamase genes, with bla_{TEM} being predominant gene detected (15.9%, 7/44). ERIC and REP-PCR analysis revealed a greater degree of genetic diversity among the ten β-lactamase producing *E. coli* isolated from Krishna River water.

Keywords: Most probable number (MPN), Coliforms, Extended Spectrum β-lactamases (ESBL's), Genetic diversity

Introduction:

Aquatic environments act as a natural territory for a wide range of microorganisms having both beneficial and pathogenic characteristics. Pathogenic bacteria are causative agents of infectious diseases in humans and aquatic animals (Gao et al., 2024). Microbial indicators are part of aquatic microflora used to determine whether or not water is safe for use. Faecal indicator bacteria have been used for more than a century to identify possible sources of faecal contamination. Among them, faecal coliform *E. coli* have been often employed to assess water quality (Holcomb and Stewart, 2020).

Total coliform bacteria define a group of gut bacteria that include *E. coli*, Klebsiella species, Enterobacter and Citrobacter species (Al-Badaii and Shuhaimi-Othman, 2015). They are gram negative, facultative anaerobic, non-spore forming motile rods, capable of fermenting lactose with production of acid and gas at 370C within 48 hours (P. Feng (ret.) et al., 2020). Even though they are generally not harmful themselves, they indicate the possible presence of other pathogenic bacteria, viruses and protozoa (Kara et al., 2004). *E. coli* is a taxonomically well-defined member of the family Enterobacteriaceae and is characterized by possessing enzymes β-galactosidase, grows at 44ºC on complex media like Endo or EMB agar, ferments lactose with production of acid and gas at 44ºC within 24-48 hours (Basavaraju and Gunashree, 2023).

E. coli is widely used as index of faecal pollution of water because it is easily detected, it is present in abundance as compared to other organisms, it survives for longer period and its source is exclusively human and animal intestine (Jang et al., 2011; Niyoyitungiye et al., 2020). Therefore, its detection is an indication of faecal pollution and its presence in drinking water indirectly indicates the presence of other members of the Enterobacteriaceae family. Shiga-like toxin-producing *E. coli* (STEC) and especially serotype O157 are important emerging pathogens that can cause a variety of clinical symptoms ranging from mild diarrhea to severe bloody diarrhea. Complications like hemolytic uremic syndrome (HUS) can be life-threatening and it is assumed that shiga-like toxins (coded by *stx* genes) are important virulence factors that play a pivotal role in the

development of HUS (Yu et al., 2021; Milton et al., 2023).

The emergence of new infectious diseases, the resurgence of several infections that appeared to have been controlled and the increase in bacterial resistance have created the necessity for studies directed towards the development of new antimicrobials (Borges et al., 2015). Because of the development of antimicrobial resistance of available antimicrobials to treat infectious diseases, many researchers have focused on the investigation of natural products as a source of new bioactive molecules. Many plant-based medicines used in conventional systems have been noted in pharmacopeia as agents used to treat diseases caused by infectious agents. Various polyphenolics derived from plant sources are reported to have antibacterial activity (Álvarez-Martínez et al., 2018; Bouarab-Chibane et al., 2019; Manso et al., 2022). Clove oil is one of the essential oils extracted from the dried flower buds of clove (Eugenia caryophyllata). The oil is rich in plant-derived phenyl propanoids, with the main compound being eugenol (Lawless, 1995). Its antimicrobial potential was established when its essential oil extracts killed many Gram-positive and Gram-negative organisms including some fungi(Nazzaro et al., 2013). The antimicrobial activity of clove is attributable to eugenol, oleic acids and lipids found in its essential oils. However, the antimicrobial activity of clove oil against MDR *E. coli* is not well established either individually or in combination with antibiotics (Nzeako et al., 2006).

Vijayawada city, a major city of the state of Andhra Pradesh, is situated on the banks of River Krishna which serves as the major lifeline in terms of supplying drinking water. So, keeping in view all these facts the current study was carried out to detect the coliform counts along the downstream, molecular characterization, antimicrobial resistance and genetic diversity in *E. coli* isolates concerning rural-urban landscape along river Krishna in Vijayawada city and screening of phytochemicals (Cinnamic acid and clove oil) for their inhibitory activity on β-lactamase producing bacteria and synergistic activity of antimicrobials in combination with phytochemicals.

Materials and Methods:

Sample collection

The study was conducted on Krishna River water to assess the extent of bacterial contamination (coliforms) from October 2018 to January 2019 in the Vijayawada city landscape. The study was conducted in the Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India. A total of 27 samples (in triplicate) from 9 different ghat locations (Figure 1) of Krishna River in Vijayawada were collected and analysed. Sampling locations were identified based on prominent activities of bathing, washing, and adding sewage/wastewater into the river. The water samples were taken from nine different Ghats. The sampling was done by immersion type of sampling method, obtaining water sample at 30 cm depth of water body, in pre-rinsed sterilized glass bottles. Around 200 ml of water sample is collected in glass bottles. During the collection of samples, extreme care was taken to avoid contamination. Water is collected from upstream with PPE including lab coat and gloves. Collection time and the site of the collection were noted on the sampling container or sampling bottle. The water collected is transported for testing immediately by ice-cold containers within 50 min of collection.

Microbiological analysis of water samples by Most probable number (MPN) technique

The enumeration of total coliforms (*Enterobacter*, *Citrobacter*, *Klebsiella* and *Escherichia*) in a water sample can be done by statistical estimation called the Most probable number (MPN) test using 3 tube method (Phyo et al., 2019). This test involves a multiple series of Durham Fermentation tubes and is divided into three parts: This test was performed according to the method given in American Public Health Association recommendations (APHA, 2005). In addition to determining the presence or absence of coliforms, the presumptive test was also used to determine the most probable number of coliforms present in the sample.

Fermentation tubes containing 10 ml of single-strength MacConkey (SSMB) and double-strength broth (DSMB) were prepared. A Durham tube was placed in each test tube in an inverted position. The tubes were labeled according to the amount of water sample to be dispensed into it i.e., 10 ml, 1 ml, and 0.1 ml. The water sample was homogenized by vigorously shaking the bottle containing the water several times. Aliquots of 10 ml of the water sample were transferred to each of the 5 DSMB tubes using a 10 ml pipette, with a micropipette, 1 ml and 0.1 ml of the water sample were transferred to batches of 5 ml SSLB tubes each (Manian et al., 1991; Li et al., 2009; A˚strömet al., 2015). The tubes were incubated at 37±1°C for 24 hrs and the number of tubes in each set that produced 10% or more gas with a change in color from pink to yellow was recorded. MPN was determined by referring to the MPN table by Mackie and McCartney (APHA, 2005).

A presumptive test was performed on all primary fermentation tubes which showed positive results with gas formation after incubation of 24 h and 48 hr periods. Fermentation tubes with brilliant green lactose bile broth (BGLB) were inoculated with culture taken from the

tubes showing a positive result in the presumptive test (Omezuruikeet al., 2008; Mishra et al., 2009; Matta, 2014; Some et al., 2021). The inoculated BGLB tubes were incubated for 48hr at 35±0.5°C. The culture from positive BGLB tubes streaked on EMB agar and incubated at 37ºC for 24 h.On EMB agar plate**,** *E. coli* colonies have a characteristic green metallic sheen.For further confirmation of *E. coli*, the biochemical tests like Triple sugar iron agar test (TSI), Oxidase test, Catalase test and IMViC tests were conducted (Chaitanya et al., 2021).

Molecular Identification and Virulence Characterization of *E. coli*

DNA was extracted from isolates with the help of boiling and snap chill method as described earlier (Bobbadi et al., 2020). *E. coli* isolated from water samples by cultural methods were confirmed using *E. coli* specific-uniplex PCR targeting *E16S rRNA* gene as described by (Sun et al., 2011). All the confirmed *E. coli* isolates from different sources were screened for the presence of virulence genes such as *stx1*, *stx2*, *eaeA* and *hlyA*genes as described by Paton and Paton (Paton and Paton, 1998). ATCC 25922 served as the positive control.

Antimicrobial resistance profiling

Antibiogram of *E. coli* isolated from different sources in the present study was carried out against 14 different clinically important antibiotics using the Kirby Bauer disc diffusion method (Bauer et al., 1966). Direct colony suspension of each isolate was made in PBS (pH 7.4) and the turbidity was adjusted to 0.5 McFarland units (equivalent to an approximate cell density of $1.5x10^8$ CFU/ml). The diameter of inhibition zones was measured and susceptibility patterns of *E. coli* species were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2019).

Phenotypic and genotypic detection of Beta-lactamase production

E. coli isolated in the present study were screened for Extended Spectrum β-lactamase production (ESBL) by examining the susceptibility of the isolates against four antimicrobial agents: cefotaxime, ceftazidime, ceftriaxone and aztreonam. After incubation for 48hr at 37ºC, resistance to at least one of the four antibiotics used in screening was considered as positive for possible ESBL production as per CLSI guidelines. All the isolates that were found to be positive in the screening test were subjected to the Phenotypic Confirmatory Test (PCTs) as recommended by CLSI guidelines. Combination Disc method was used in the present study. HexaGminus 24 discs supplied by M/s HiMedia (with and without β-lactamase inhibitor) containing ceftazidime, ceftazidime plus clavulanic acid, cefotaxime, cefotaxime plus clavulanic acid, cefpodoxime, cefpodoxime plus clavulanic acid were placed on the surface of agar MHA plate inoculated with *E. coli* isolates. Following incubation at 37ºC for 24h under favorable conditions, the resistant zone diameter around the combination antibiotic discs was $\geq$ 5mm when compared to discs containing cephalosporin (respective) alone, then the isolate was confirmed to be phenotypically positive for the respective β-lactamase production (Drieux et al., 2008). *E. coli* ATCC 25922 and K. pneumoniae ATCC 700603 were used as beta-lactamase negative and positive controls, respectively for phenotypic screening and molecular confirmatory tests of ESBL production. Molecular screening for beta-lactamase genes was done with the help of primer sets published earlier (Dallenne et al., 2010; Manoharan et al., 2012) mentioned in the Tables 1 and 2.

Assessment of genetic diversity

Assessment of the genetic relatedness among the ESBL-producing *E. coli* isolates was performed using PCR-based techniques such as ERIC-PCR and REP-PCR. ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') primer pair was used for the amplification of conserved ERIC sequences in the chromosomal DNA of *E. coli* isolates. Further *E. coli* isolates were subjected to REP-PCR fingerprinting using a single oligonucleotide primer (GTG)(5'-GTGGTGGTGGTGGTG-3') as described by Mohapatra et al. (2007) with slight modifications. The discriminatory power using Simpson's diversity index was calculated with the help of the online tool (http://insilico.ehu.eus/mini_tools/discriminatory_power/)

Minimal inhibitory concentration (MIC) of phytochemical (clove oil) against MDR *E. coli* isolates

Preparation of stock solution

The stock solution of Clove oil was prepared by dissolving $\cong$ 16 µl of clove oil in 1 ml of DMSO to obtain a final concentration of 10 mg/ml stock concentration for the test compounds. The stock solution was kept in screw-capped bottles for subsequent use. Working dilutions were made in 1% DMSO. The stock solutions of penicillin, gentamicin, and enrofloxacin were $1mg.ml^{-1}$, 5 mg ml^{-1} and 1 mg ml^{-1} respectively.

Bacterial cultures

MDR *E. coli* isolates viz PB1 and KR2 isolates were used for the present study. The isolates were cultured onto nutrient agar and incubated at 37°C for 16-18 h. Isolated colonies were selected and inoculated into Muller-Hinton broth before use in the microdilution assay (Tukmechi et al., 2010).

Determination of minimum inhibitory concentration (MIC)

The MIC of individual polyphenolic compounds was determined using serial microplate dilution method of Eloff (1998) with few modifications. Two-fold serial dilutions of test compounds (100 µl) in sterile cation-adjusted Muller Hington broth were prepared in a 96-well microtitre plate. Ten µl of 0.5 McFarland unit adjusted overnight fresh bacterial cultures of MDR *E. coli* isolates viz PB1 and KR2 isolates were added to each well. Resazurin (2 mg/ml) was added to each well to know the bacterial growth (25 µl). The plates were incubated overnight at 37ºC. After incubation at 37ºC, resazurin is reduced to a violet formazan by biologically active organisms. The development of pink color indicates bacterial growth. Bacterial growth is inhibited when the solution in the well remains violet in color. This concentration was taken as the minimum inhibitory concentration (MIC). Solvent controls were included in each experiment.

Results and Discussion:

Most probable number (MPN) of water samples

This study investigated the water quality of River Krishna flowing along the sides of Vijayawada city with emphasis on MDR and ESBL producing *E. coli*. The coliform count was evaluated by the MPN method. This method is based on the bacteria's ability to produce gas when grown in a MacConkey broth medium at 37°C. The MPN values for coliforms ranged between 6 and 2400/100 ml (Table 3). Cultural and biochemical tests were done to identify the *E. coli* isolates

Genotypic characterization results of *E. coli*

Screening of the water samples after initial cultural methods and confirmation by *E. coli*-specific PCR revealed that 44 out of 54 isolates were positive for *E. coli*, yielding a 231bp product of E16S rRNA gene, with an overall occurrence rate of 81.48% (68.57 – 90.75%, 95% confidence interval). The positive samples were further subjected to m-PCR targeting *stx*1, *stx*2, *eae*A, and *hly*A genes for identification of STEC. Out of 44 isolates two isolates carried all four genes, one isolate harbored stx_2, *eaeA,* and*hlyA*genes and one isolate showed the presence of *stx*1and*hlyA*genes in combination (Figure 2 and Table 1). The occurrence of STEC was found to be 7.41% (2.05 – 17.89%, 95% confidence interval).

Antimicrobial resistance of isolated *E. coli*

All 44 *E. coli* isolates from different ghats were subjected to antibiotic sensitivity tests using 12 different and most commonly used antibiotics in veterinary medicine as mentioned. Interestingly higher resistance was observed in nalidixic acid (40.9%) followed by ampicillin (38.63%), ceftazidime (34%), ceftriaxone (27.27%), aztreonam (25%), tetracycline (20.45%), ciprofloxacin (18.18%), co-trimoxazole (67%), lower resistance to nitrofurantoin (8%), amikacin (13.66%), gentamicin (4.5%) and chloramphenicol (4.5%) (Table 4).

Phenotypic and molecular characterization of Extended Spectrum β-lactamase production

E. coli isolated in the present study were screened for Extended Spectrum β-lactamase production (ESBL) production by examining the susceptibility of the isolates against four antimicrobial agents: cefotaxime, ceftazidime, ceftriaxone, and aztreonam. All the isolates that were found to be positive in the screening test were subjected to the Phenotypic Confirmatory Test (PCT) as recommended by CLSI (Lewis et al., 2023) guidelines as well as for molecular confirmation. A total of 10 isolates (22.73%, 11.47% – 37.84%, 95% CI) were found to be positive for ESBL production by CDM (confirmatory disc method). Out of 10 CDM-positive isolates of *E. coli*, all isolates harbored β-lactamase genes, with bla_{TEM} being predominant gene detected (15.9%, 7/44), followed by $bla_{\text{CTX-M}}$ group 1 (9%, 4/44), $bla_{\text{CTX-M}}$ group 2 (6.8%, 3/44), bla_{SHV} (4.5%, 2/44), bla_{ACC} (2.2%, 1/44), bla_{MOX} (2.2%, 1/44) and one bla_{IMP} gene (2.2%, 1/44). The predominance of the bla_{TEM}gene in ESBL-positive *E. coli* isolates has been reported earlier (Vaiyapuri et al., 2021); however, the occurrence rate was comparatively higher (83.3%).

Genetic diversity of MDR *E. coli* isolates

A total of 10 MDR *E. coli* isolates and one MTCC strain (*E. coli* ATCC 25922) were subjected to DNA-based fingerprinting by two methods (ERIC and REP-PCR) to differentiate them based on genetic diversity and to assess the intra-specific diversity. ERIC-PCR and REP-PCR fingerprinting profiles were visualized under UV Transilluminator, photographed, and compared for similarity by visual inspection of the band profiles as well as by using image lab software (BIO-RAD). Both ERIC and REP-PCR sequences were found to be present in 10 MDR *E. coli* isolates. Patterns with at least one different band were considered different genotypes. In other words, isolates that had patterns showing the same number of bands with the same size of corresponding bands were considered indistinguishable or the same genotype. Dendrograms were constructed for the MDR *E. coli* isolates from the binary scores obtained from ERIC and REP-PCR fingerprint data.

ERIC-PCR typing revealed 3-11 fragments per isolate, ranging in size from ~100 bp to ~2000 bp, whereas REP-PCR typing revealed 3-12 fragments resolved per isolate, ranging in size from ~100 bp to ~2000 bp. Of the 10 MDR *E. coli* analyzed, 10 ERIC-PCR patterns and 10 REP patterns were obtained with a discriminatory power

1 and typeability 100%. Dendrogram analysis of ERIC-PCR profiles discriminates isolates into two major clusters (Figure 3). Cluster I comprised two isolates KR2 and PB1 were obtained from Krishna Ghat and Prakasam barrage, respectively showing genetic relatedness. In cluster II, *E. coli* ATCC 25922 was closely clustered with an isolate of Godavari ghat origin (GOD2) showing 90% similarity cut-off. Within cluster II, the isolate from Krishna ghat (KR4) is distantly related to the other two isolates. The remaining seven isolates (G5, G2, KR1, BH2, B5, and PB4) were found to be unclustered (UC) with other isolates.

Dendrogram analysis of REP-PCR profiles discriminated MDR *E. coli* isolates into a 3 major clusters (Figure 4). Cluster I has 2 isolates (KR4 and G5) that were distantly separated from *E. coli* ATCC 25922. p53 isolate was distantly related. Cluster II has three isolates (GOD2, B5, and G3) whereas Cluster III has two isolates (KR1 and PB4) obtained from different ghats. Three isolates (BH2, KR2, and PB1) were found to be unclustered (UC) with other isolates. Cluster analysis of ERIC-PCR and REP-PCR indicated wide genetic diversity among the isolates.

Minimal inhibitory concentration (MIC) of phytochemical (Clove oil) against MDR *E. coli* isolates

The inhibitory activity of phytochemicals on β-lactamase production was studied. The isolates were subjected to Clove oil treatment using MIC (microtiter plate assay) along with antibiotics. Synergistic activity of clove oil along with antibiotics was observed. Both MDR *E. coli* isolates showed sensitivity to clove oil and they also showed synergistic activity with antibiotics. The mean MIC values for individual agents are expressed as the Mean of 6 observations (Table 1). The MICs of tested compounds i.e. penicillin, gentamicin, enrofloxacin, and clove oil against both MDR *E. coli* isolates were in the range of 0.95 $ng.ml^{-1}$ to 3 $\mu g.ml^{-1}$. Among tested antibiotics penicillin showed the lowest MIC (0.030 $\mu g.ml^{-1}$) against KR2 isolate however enrofloxacin showed the highest MIC (3 $\mu g.ml^{-1}$) against PB1 isolate respectively. Similarly, the KR2 isolate was more sensitive to clove oil (MIC: 0.0038 $\mu g.ml^{-1}$). MIC of all the tested antibiotics against both the isolates were significantly reduced in combination with clove oil however penicillin showed more reduction in MIC (0.95 $ng.ml^{-1}$) value against PB1 isolate when combined with clove oil (Table 5 and Figure 5 and 6).

The river Krishna exhibited a high density of faecal coliforms at selected sites. The MPN/100 mL values of faecal coliforms recorded at all the sampling sites exceeded the standards set by regulatory authorities for surface water reservoirs to be used for drinking and recreational purposes (WHO, 2002). These ghat sites are highly polluted due to recurring faecal pollution from nearby residential, holy dips performed by pilgrims, entry of polluted city wastewater or entry of untreated sewage water from the city, and cremation practices at banks of the river. Population pressure and poor sanitation facilities have been responsible for an increased presence of pathogenic organisms exhibiting virulence genes in surface waters of developing countries (Ram and Shanker, 2005; Hamner et al., 2007; Singh et al., 2021, 2024). According to results obtained from MPN of water samples, all the samples collected from different Ghats were unsafe for direct drinking. The increased pattern of MPN index value (6-2400 per 100ml) was recorded from Godavari ghat to Pedapulipaka ghat.

Diarrheal diseases in humans due to consumption of STEC-contaminated surface waters have been reported in Bangladesh, India, and South Africa (Hamner et al., 2007; Hossain et al., 2021; Robert et al., 2021). The prevalence of virulence markers *stx1* and *stx2* together or *stx2* alone in STEC isolates, respectively, indicates faecal pollution of animal and human origin in the river Krishna. A few research studies reported the contamination of river water in India by STEC (Hamner et al., 2007; Ram et al., 2007). Further, the presence of STEC exhibiting both *stx1*and *stx2* has also been reported in human and cattle stool samples from Kashmir, West Bengal, and Karnataka in India (Khan et al., 2002; Wani et al., 2006; Kumar et al., 2004, 2012). STEC has a high rate of infection and person-to-person transmission due to a very low infectious dose of 1-10 colony-forming units with a short incubation period of 3 Hr (Kuhnert et al., 2000). Amid the present investigation, all STEC isolates possess *eaeA* gene along with *stx1* and *stx2* or *stx2* alone. The presence of *eaeA* gene in these isolates increases virulence as this gene is required for the expression of virulence of STEC isolates in human beings leading to hemorrhagic colitis and hemolytic uremic syndrome (Amin et al., 2022). The virulence-associated factor hemolysin (*hlyA*) known to be encoded on a plasmid was observed to be the most prevalent gene in STEC isolates from the river Ganga (Ram et al., 2007). The *hlyA* gene was also found to be present in all isolates of STEC recovered from the river Krishna.

The administration of antibiotics during the management of STEC infections is not recommended as it may complicate disease progression by the release of Shiga toxins in vivo through bacterial cell lysis, finally leading to host cell death. However, recent therapeutic options recommend antimicrobial therapy in STEC infections combined with oral administration of Shiga toxin-binding or inactivating agents (MacConnachie and Todd, 2004). The emergence of resistance to multiple antimicrobials in STEC isolates observed from our research might be attributed to the treatment of diarrheal diseases in India and other developing countries by inadequate quantity of

antimicrobials without identifying pathogens (Ram et al., 2007). Hence, the dissemination of resistance to antimicrobials among STEC isolates may have potential negative clinical implications for therapeutic advancement for the management of STEC infections during epidemics. This may also lead to clinical complications by limiting the number of antibiotics for use in the treatment of diarrheal diseases during waterborne outbreaks. Multi-antimicrobial resistant *E. coli* has been detected in surface water samples of developing and developed countries (Edge and Hill, 2005; Qadri et al., 2005; Hamelin et al., 2006; Jianying et al., 2008). Our study indicates that the river Krishna flowing through Vijayawada city is a reservoir of multiple antimicrobial-resistant *E. coli* and 46.7% of *E. coli* isolates recovered in the present study exhibited resistance to three or more antimicrobials. The results in the present study are more or less similar to the earlier findings (Begum et al., 2005; Ram and Shanker, 2005; Ram et al., 2007; Li et al., 2009).

ESBL-producing *E. coli* are considered good indicators for the surveillance of AMR (World Health Organization, 2021). The results of presnt study is similar rates (12%) were reported from a study in Central India (Diwan et al., 2018); however, a higher rate of phenotypic ESBL producers (44.57%) was reported from a study on water from Vembanad Lake of India (Vaiyapuri et al., 2021).

ERIC and REP PCR indicated possible intermixing of water sources from different areas. Microbial source tracking using repetitive elements has been employed to good use in earlier studies conducted in Canada and India with a satisfactory discriminatory power (> 0.9) (Mohapatra et al., 2007; Vaiyapuri et al., 2021).

Many plant-based medicines used in conventional systems have been noted in pharmacopeia as agents used to treat diseases caused by infectious agents. Various polyphenolics derived from plant sources are reported to have antibacterial activity. Clove oil is one of the essential oils extracted from the dried flower buds of clove (*Eugenia caryophyllata*)**.** The oil is rich in plant-derived phenyl propanoids, with the main compound being eugenol (Lawless, 1995). Many authors regarding the antimicrobial activity of clove oil against gram-positive reported similar findings and gram-negative micro-organisms (Nzeako et al., 2006) and many have shown that the antimicrobial property of clove oil is mainly because of the presence of eugenol (Bai et al., 2023).

Conclusions:

The present study warrants that all the samples collected from different Ghats of Vijayawada were unsafe. The increased pattern of MPN index value (6-2400 per 100ml) was recorded from Godavari ghat to Pedapulipaka ghat. *E. coli* isolated were β-lactamase producers and were resistant to all the antibiotics used in field conditions. The synergistic activity of clove oil along with antibiotics on MDR *E. coli* was observed. ERIC and REP-PCR analysis revealed a greater degree of genetic diversity among the ten β-lactamase-producing *E. coli* isolated from Krishna River water. Thus, from the present study, it can be concluded that water samples collected from different ghats of Vijayawada city landscape were contaminated with multi-drug-resistant *E. coli* harbouring clinically important resistance genes.

Conflict of Interest:

The authors declare no conflict of interest.

Author Contributions:

Chaitanya Gottapu: Conceptualization; investigation; writing- original draft; formal analysis.Ch. Bindu Kiranmayi: Conceptualization; writing- original draft; funding; resources. T. Srinivasa Rao: writing- reviewing and editing; resources. K. Aswani Kumar: Writing-reviewing and editing; formal analysis. Afroz Jahan: formal investigation; writing -original draft; Phytochemical resaerch. N. Subhashini: writing-reviewing and editing, B. Swathi Vimala: formal investigation, writing -original draft. Pranaw Sinha: Stastical analysis and reviewing, Srinivas K: Writing - reviewing and editing.

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Table 1- Primers used for the detection of Shiga toxigenic *E. coli*

Target genes	Primers	Primer sequence	Product size (bp)
E16S rRNA	*E16S*: F	ATCAACCGAGATTCCCCCAGT	231
	E16S: R	TCACTATCGGTCAGTCAGGAG	
stx 1	*stx* 1: F	ATAAATCGCCATTCGTTGACTAC	180
	stx 1: R	AGAACGCCCACTGAGATCATC	
stx 2	*stx* 2: F	GGCACTGTCTGAAACTGCTCC	255
	stx 2: R	TCGCCAGTTATCTTGACATTCTG	
eaeA	*eaeA*: F	GACCCGGCACAAGCATAAGC	384
	eaeA: R	CCACCTGCAGCAACAAGAGG	
hlyA	*hlyA*: F	GCATCATCAAGCGTACGTTCC	534
	hlyA: R	AATGAGCCAAGCTTGTTAAGCT	

Table 2- Primers used for the detection of beta-lactamase genes (34,35)

Primer	Sequence (5-3)	Amplicon size
TEM: F	CATTTCCGTGTCGCCCTTATTC	800
TEM: R	CGTTCATCCATAGTTGCCTGAC	
SHV: F	AGCCGCTTGAGCAAATTAAAC	713
SHV: R	ATCCCGCAGATAAATCACCAC	
OXA: F	GGCACCAGATTCAACTTTCAAG	564
OXA: R	GACCCCAAGTTTCCTGTAAGTG	
CTX-M-1:F	TTAGGAARTGTGCCGCTGYA	688
CTX-M-1:R	CGATATCGTTGGTGGTRCCAT	
CTX-M-2: F	CGTTAACGGCACGATGAC	404
CTX-M-2:R	CGATATCGTTGGTGGTACCAT	
ACC-F	AACAGCCTCAGCAGCCGGTTA	346
ACC-R	TTCGCCGCAATCATCCCTAGC	
MOX-F	GCTGCTCAAGGAGCACAGGAT	520
MOX-R	CACATTGACATAGGTGTGGTGC	
FOX-F	AACATGGGGTATCAGGGAGATG	190
FOX-R	CAAAGCGCGTAACCGGATTGG	
DHA-F	AACTTTCACAGGTGTGCTGGGT	405
DHA-R	CCGTACGCATACTGGCTTTGC	
CIT-F	TGGCCAGAACTGACAGGCAAA	462
CIT-R	TTTCTCCTGAACGTGGCTGGC	
EBC-F	TCGGTAAAGCCGATGTTGCGG	302
EBC-R	CTTCCACTGCGGCTGCCAGTT	
IMP-F	TTGACACTCCATTTACDG	139
IMP-R	GATYGAGAATTAAGCCACYCT	
VIM-F	GATGGTGTTTGGTCGCATA	390
VIM-R	CGAATGCGCAGCACCAG	
KPC-F	CATTCAAGGGCTTTCTTGCTGC	538
KPC-R	ACGACGGCATAGTCATTTGC	

Table 3: MPN counts of Coliform from Krishna River water at different Ghats of Vijayawada		
S.No	**GHAT**	**MPN VALUE**
1	GODAVARI GHAT	0-3-0 (6) 2-3-0 (12) 3-5-0 (25)
2	KRISHNA PUSHKAR GHAT	3-2-2 (20) 1-2-2 (10) 3-5-0 (25)
3	PAVITRASANGAMAM GHAT (FERRI)	5-5-1 (350) 3-5-0 (25) 0-3-0 (6)
4	ARIHANTH GHAT	3-4-1 (24) 3-0-0 (8) 3-2-2 (20)
5	PUNNAMI GHAT	3-4-1 (24) 3-5-0 (25) 2-3-0 (12)
6	K. L. RAO GHAT	5-5-4 (1600) 5-4-5 (430) 5-4-3 (280)
7	KRISHNAVENI GHAT	4-3-2 (39) 5-4-3 (280) 5-5-5 (2400)
8	VIJAYAKRISHNA PUSHKAR GHAT	5-5-5 (2400) 5-5-5 (2400) 5-5-5 (2400)
9	PEDAPULIPAKA GHAT	5-5-5 (2400) 5-5-4 (1600) 5-5-5 (2400)

Table 4: *In vitro* antimicrobial susceptibility testing				
S No.	**Antimicrobial agent (Dose)**	**Antibiogram pattern**		
		Sensitive No. (%)	**Intermediate No. (%)**	**Resistant No. (%)**
1	Amikacin (30µg)	23 (52.27%)	15 (34.09%)	6 (13.63%)
2	Aztreonam (30µg)	27 (61.36%)	6 (13.63%)	11 (25%)
3	Ampicillin (10µg)	21 (47.72%)	6 (13.63%)	17 (38.63%)
4	Ceftazidime (30µg)	17 (38.63%)	12 (27.27%)	15 (34.09%)
5	Ceftriaxone (30µg)	27 (61.36%)	5 (11.36%)	12 (27.27%)
6	Chloramphenicol (30µg)	39 (88.63%)	3 (6.81%)	2 (4.5%)
7	Ciprofloxacin (5µg)	23 (52.27%)	13 (29.54%)	8 (18.18%)
8	Co-Trimoxazole (25µg)	33 (75%)	4 (9%)	7 (15.9%)
9	Gentamicin (10µg)	37 (84.09%)	5 (11.36%)	2 (4.5%)
10	Nalidixic acid (30µg)	1 (2.27%)	3 (6.81%)	40 (90.90%)
11	Nitrofurantoin (300µg)	31 (70.45%)	7 (15.9%)	6 (13.63%)
12	Tetracycline (30µg)	35 (79.54%)	---	9 (20.45%)

Table 5: MICs of antibiotics and clove oil individually and in combination µg.ml^{-1}		
Compounds	**PB1 isolate**	**KR2 isolate**
Penicillin	0.975	0.0305
Gentamicin	0.305	0.0763
Enrofloxacin	3.0	0.244
Clove oil	0.0305	0.0038
Penicillin + Clove oil	0.00095	0.0076
Gentamicin + Clove oil	0.019	0.00238
Enrofloxacin + Clove oil	0.0019	0.0038

Figure 1: Water sample collection sites from different Ghats of Krishna River along Vijayawada city landscape (Credits: Google maps)

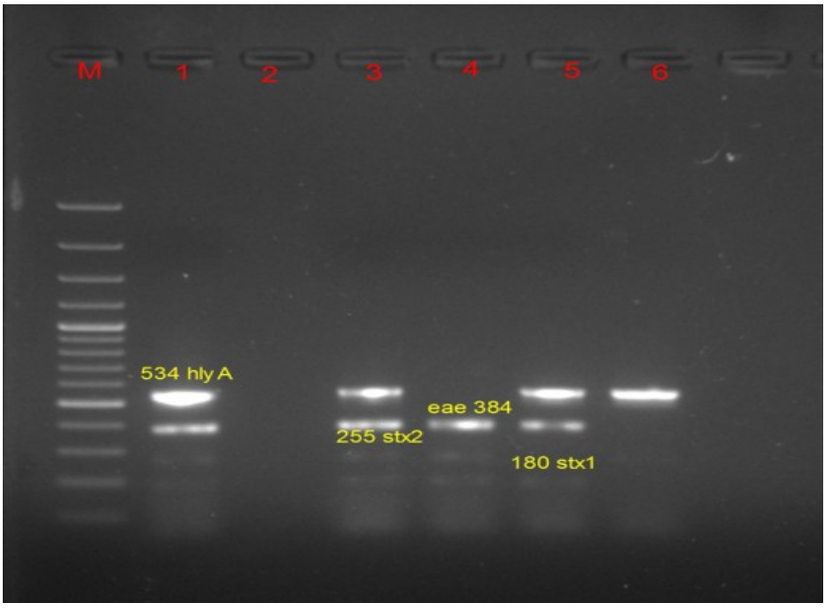

Figure 2: Gel photograph of *E. coli* virulent genes
Lane M: DNA ladder (100bp)
Lane 1: Standard DNA *E. coli* (ATCC 25922) showing 4 genes
Lane 2: Negative control
Lane 3: Amplified DNA of *E. coli* showing 4 genes
Lane 4-5: Amplified DNA of *E. coli* showing 3 genes
Lane 6: Amplified DNA of *E. coli* showing 2 genes

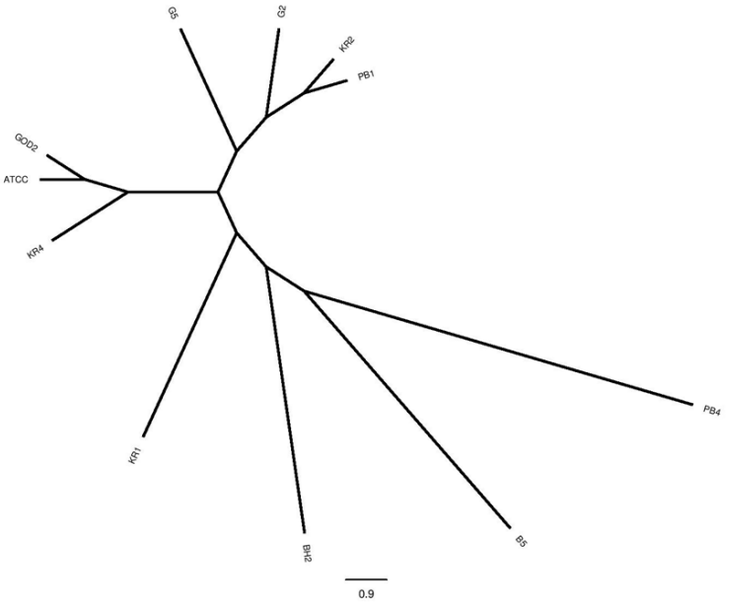

Figure 3: ERIC PCR Dendrogram analysis

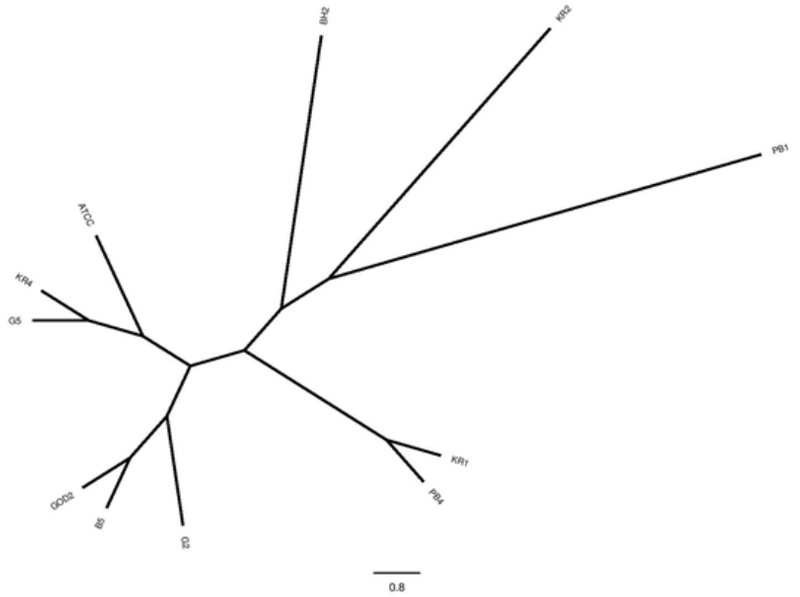

Figure 4: REP PCR Dendrogram analysis

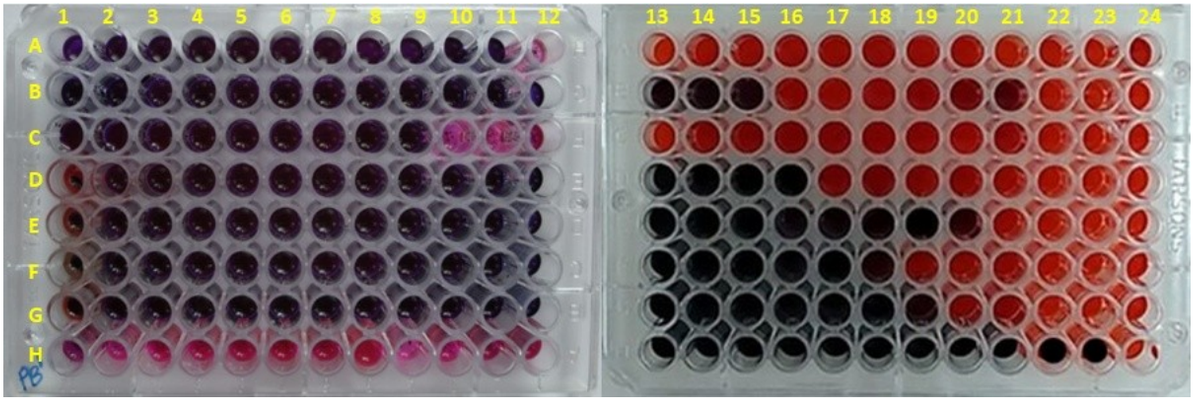

Figure 5: Minimum inhibitory concentration (MIC) of polyphenolic compounds (µg. ml^{-1}) of PB1 isolate

Row A: Penicillin in serial dilution + broth culture+ indicator
Row B: Gentamicin in serial dilution + broth culture+ indicator
Row C: Enrofloxacin in serial dilution + broth culture + indicator
Row D: Clove oil in serial dilution + broth culture + indicator
Row E: Clove oil and Penicillin (1:1) in serial dilution + broth culture + indicator
Row F: Clove oil and Gentamicin (1:1) in serial dilution + broth culture + indicator
Row G: Clove oil and Enrofloxacin (1:1) in serial dilution + broth culture + indicator
Row H: 1-6 wells: Broth control; 6-12 wells: 1% DMSO+ broth; 13-18 wells: Culture control; 19-24 wells :- 1% % DMSO+ Culture

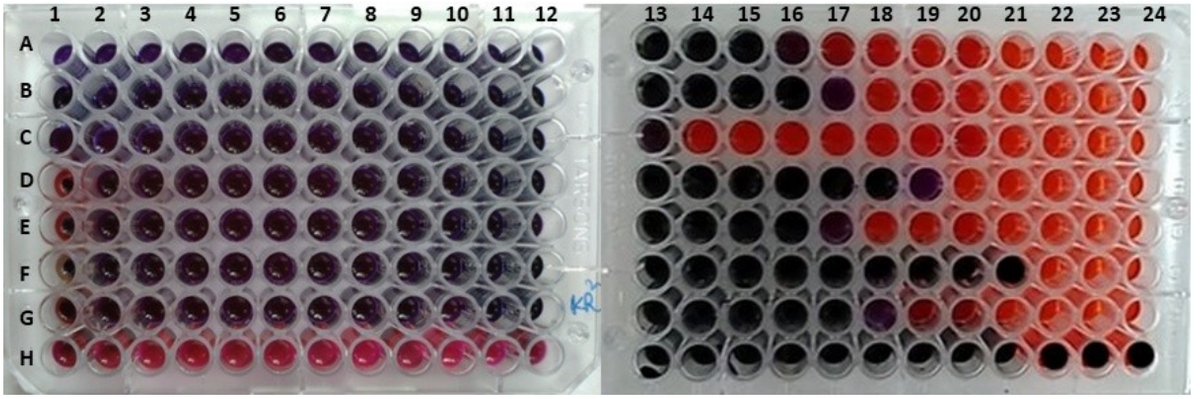

Figure 6: Minimum inhibitory concentration (MIC) of polyphenolic compounds (µg. ml^{-1}) of KR2 isolate
Row A: Penicillin in serial dilution + broth culture+ indicator
Row B: Gentamicin in serial dilution + broth culture+ indicator
Row C: Enrofloxacin in serial dilution + broth culture + indicator
Row D: Clove oil in serial dilution + broth culture + indicator
Row E: Clove oil and Penicillin (1:1) in serial dilution + broth culture + indicator
Row F: Clove oil and Gentamicin (1:1) in serial dilution + broth culture + indicator
Row G: Clove oil and Enrofloxacin (1:1) in serial dilution + broth culture + indicator
Row H: 1-6 wells: Broth control; 6-12 wells : 1% DMSO+ broth; 13-18 wells: Culture control; 19-24 wells :- 1% % DMSO+ Culture

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