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***Research Article** (Open Access)*

ANTIBIOTIC SUSCEPTIBILITY PROFILING OF EXTENDED-SPECTRUM B-LACTAMASE (ESBL)-PRODUCING *ESCHERICHIA COLI* ISOLATED FROM SEWAGE WATER USING THE AUTOMATED VITEK-2 SYSTEM

Vandana Jhalora and Renu Bist*
Department of Zoology, Centre of Advanced Studies, University of Rajasthan, Jaipur 302004, India
**Author for Correspondence: renu_bisht22@yahoo.co.in*

ABSTRACT
Multidrug resistance is rising at an alarming rate, with one of the primary contributing factors being the production of Extended-Spectrum β-Lactamases (ESBLs). This study investigates the prevalence of ESBL-producing *E. coli* in sewage water, particularly near healthcare facilities, and evaluates their antibiotic resistance using the automated VITEK-2 system. The *E. coli* isolates were detected using eosin methylene blue agar whereas the presence of ESBL enzymes was detected using the HiCrome™ ESBL agar and Hexa G-minus 23 antibiotic ring. The antibiotic susceptibility of the isolates was tested using the automated VITEK-2 system. Out of 157 colonies examined, 43 were confirmed as *E. coli* isolates, representing 27.4% of the total. Among these, 23.25% were identified as ESBL producers using HiCrome™ ESBL agar. A total of 10 *E. coli* isolates exhibited ESBL production, as indicated by reduced zones of inhibition (ZoI) against Hexa G-minus 23 antibiotic ring. Additionally, 100% resistance was observed for thirteen antibiotics, accounting for 43.3% of the total antibiotics tested which indicates alarming level of multidrug resistance. The study highlights a significant prevalence of ESBL-producing *E. coli* in sewage water, with a high level of resistance to multiple antibiotics, posing a public health risk. These findings underscore the need for continuous monitoring and effective strategies to combat antibiotic resistance.

Keywords: *Antibiotic susceptibility, Escherichia coli, Extended-Spectrum β-Lactamases, Multidrug resistance, VITEK-2*

INTRODUCTION
Escherichia coli (*E. coli*), a gram-negative bacterium (GNB), is a key indicator of faecal contamination and a growing concern due to its production of extended-spectrum β-lactamase (ESBL), which confers resistance to critical antibiotics (Akpaka et al., 2021). ESBL-producing *E. coli* (ESBL-EC) poses significant public health risks, particularly in wastewater, a major reservoir for antibiotic-resistant bacteria (ARB) (Davidova-Gerzova et al., 2023). Incomplete removal of ARB by wastewater treatment plants exacerbates the spread of antimicrobial resistance (AMR) (Osińska et al., 2023). The VITEK-2 system, an advanced automated platform, enables rapid and accurate detection of ESBL-EC, facilitating high-throughput monitoring (Leverstein-van Hall et al., 2002). This technology is vital for assessing AMR prevalence in environmental samples and guiding effective mitigation strategies (Kasanga et al., 2024).

MATERIALS AND METHODS
Eosin-methylene blue (EMB) agar (M317), HiCrome™ ESBL agar base (M1829), HiCrome™ ESBL agar supplement (FD278), Hexa G-minus 23 antibiotic ring, Mueller Hinton agar (MHA), Nutrient agar, and VITEK-2 automated system. All the chemicals were procured from Himedia (Chandigarh, India).

1.1 Isolation and detection of *E. coli* bacteria
Bacterial samples were collected from sewage water near hospital settings in Jaipur, Rajasthan. These samples were serially diluted and inoculated onto nutrient agar plates, which were then incubated at 37°C for 24 hours to allow for the development of distinct bacterial colonies. These distinct colonies were

subsequently streaked onto EMB agar, a medium specific for the growth of *E. coli*. The EMB agar plates were incubated for an additional 24 hours at 37°C to facilitate the identification and isolation of *E. coli* colonies.

1.2 Phenotypic detection of ESBL-positive *E. coli*

1.2.1 By chromogenic agar- The phenotypic detection of ESBL-positive *E. coli* isolates was performed using HiCrome™ ESBL chromogenic agar (Souverein et al., 2016). The medium was prepared following the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 minutes. Chromogenic agar was employed to distinguish ESBL-producing organisms based on their color. Instead of direct plating, the isolates were first cultured in LB broth at 37°C for 24 hours to achieve a turbidity equivalent to the 0.5 McFarland standard. Following this, 100 microliters of the bacterial suspension were evenly spread onto the solidified agar and incubated at 37°C for 24 hours.

1.2.2 By Hexa G-minus 23 antibiotic ring

These are the inert flat circular rings having six discs of 6 mm diameter on its projections. These discs are coated with antibiotics that aid the phenotypic confirmation of ESBL producers. It is composed of aztreonam (AT) (30 µg), cefpodoxime (CPD) (10 µg), cefpodoxime/clavulanic acid (CCL) (10/5 µg), ceftazidime (CAZ) (30 µg), ceftriaxone (CTR) (30 µg), and cefotaxime (CTX) (30 µg). The bacterial isolates were aseptically inoculated into autoclaved LB broth and incubated at 37°C for 24 hours at 150 rpm in a shaker incubator. Following incubation, the bacterial growth was standardized to a 0.5 McFarland standard. MHA plates were prepared, allowed to solidify at room temperature, and then swabbed with the standardized inoculum using a sterile, autoclaved cotton swab. After allowing the inoculum to absorb for 5-15 minutes with the lid closed, a sterile Hexa G-minus 23 antibiotic ring was aseptically placed at the center of each plate. The plates were incubated at 37°C for 24 hours, and the zones of inhibition (ZOI) were measured using an antibiotic zone scale.

1.3 Antibiotic susceptibility test (AST) by VITEK-2

All isolates were inoculated into autoclaved LB broth, incubated at 37°C for 24 hours with shaking at 150 rpm, and standardized to a 0.5 McFarland standard. The broth microdilution method was used for VITEK-2, according to CLSI guidelines (2024). Based on the organism type and antibiotics to be tested, appropriate identification (ID) cards were selected. The prepared inoculum was transferred into the cards using a sterile transfer tube, which was then loaded into the VITEK-2 compact instrument (MICROPRO™~MIC V-2.A) with software MICROPRO™~ASTRA V-2.1. The system automatically sealed and loaded the cards into the incubator/reader module, where they were incubated at 35-37°C and monitored for biochemical reactions to determine ID or growth patterns in the presence of antibiotics for AST.

RESULTS

This study aimed to identify the presence of ESBL-producing *E. coli* in sewage samples collected from areas surrounding hospitals in Jaipur, Rajasthan. The resistance profiles of all ESBL-positive *E. coli* isolates were analyzed using the automated VITEK-2 system. The findings revealed a concerning prevalence of these antibiotic-resistant strains in wastewater, particularly near healthcare facilities, highlighting a significant public health threat.

1.4 Isolation and detection of *E. coli* bacteria

Out of the total 157 colonies examined, 43 colonies were confirmed as *E. coli* isolates, accounting for approximately 27.4% of the total colonies. The identification was based on the distinctive metallic green sheen exhibited by the colonies when cultured on EMB agar (figure 1).

1.5 Phenotypic detection of ESBL-positive *E. coli*

1.5.1 By chromogenic agar- In this study, out of the total *E. coli* isolates, 23.25% were identified as ESBL producers using HiCrome™ ESBL agar, which is a selective chromogenic medium designed to differentiate between ESBL-producing and non-ESBL-producing bacteria. The characteristic purple-

coloured colonies observed on the HiCrome™ ESBL agar confirmed the presence of ESBL-positive *E. coli* (figure 2).

1.5.2 By Hexa G-minus 23 antibiotic ring- A total of 10 *E. coli* isolates exhibited ESBL production as the zone of inhibition (ZoI) against the CCL, CAZ, CTR, CTX, AT, and CPD antibiotics were less than the standard values according to CLSI guidelines (figure 3). All the 10 ESBL positive isolates were highly resistant to CTR, and CPD as none of them showed the inhibition. Although, 100% resistance rate was observed for all the six antibiotics present in the ring. All the results are summarized in table 1. According to CLSI guidelines, *Escherichia coli* is considered as ESBL positive if their zones of inhibition (ZOI) are as follows:

Antibiotic	**ZOI**
CPD (10 µg)	≤ 17 mm
CAZ (30 µg)	≤ 22 mm
AT (30 µg)	≤ 27 mm
CTX (30 µg)	≤ 27 mm
CTR (30 µg)	≤ 25 mm

1.6 AST by VITEK-2

The results obtained from the VITEK-2 system provide a detailed analysis of the antimicrobial susceptibility profile of ESBL-positive *E. coli*. Utilizing advanced technology, the system ensures precise detection of both resistance and sensitivity patterns, offering critical support for clinical decision-making. The AST conducted through VITEK-2 revealed a concerning pattern of alarming levels of resistance to multiple antibiotics. Out of the thirty antibiotics tested against the ESBL-positive *E. coli* strain, 100% resistance was recorded for thirteen antibiotics (43.3%): ampicillin, ampicillin/sulbactam, cefazolin, cefepime, cefixime, cefotaxime, cefoxitin, ceftazidime, cefuroxime, chloramphenicol, colistin, levofloxacin, and norfloxacin. High resistance rates were also noted for nitrofurantoin (90%), ciprofloxacin, ertapenem, and polymyxin B (80%), meropenem, piperacillin/tazobactam, and tetracycline (70%). A moderate resistance level was documented for amikacin (60%). In contrast, only three antibiotics-gentamicin, minocycline, and trimethoprim/sulfamethoxazole exhibited complete sensitivity (100%), representing just 10% of the total antibiotics tested. Nalidixic acid and tobramycin showed 70% sensitivity. The sensitivity rate for fosfomycin was recorded to be 60%. The remaining antibiotics demonstrated variable resistance and sensitivity patterns, emphasizing the importance of precise antimicrobial susceptibility testing for effective therapeutic management. The minimum inhibitory concentration (MIC) breakpoints given by CLSI were used to determine whether the ESBL positive *E. coli* is susceptible, intermediate, or resistant to various antibiotics (table 2).

DISCUSSION

In this study, 43 colonies out of the 157 examined (27.4%) were confirmed as *E. coli* isolates based on their characteristic metallic green sheen on EMB agar. This aligns with previous findings demonstrating the efficacy of EMB agar in isolating *E. coli* from mixed microbial samples, as the green sheen indicates lactose fermentation and acid production (Antony & Silvester, 2016). The recovery rate of 27.4% in this study is consistent with earlier reports of comparable prevalence rates from environmental samples (Kasanga et al., 2024). The reliance on EMB agar as a selective medium stems from its ability to inhibit the growth of Gram-positive organisms while promoting differentiation of lactose-fermenting GNB like *E. coli* (Leininger et al., 2001). Similar research conducted from environmental water samples reported an *E. coli* prevalence of 26.7%, closely matching the findings here (Akbar et al., 2022). Such similarities underscore the adaptability and widespread distribution of *E. coli* in diverse settings.

In this study, 23.25% of the *E. coli* isolates were identified as ESBL producers using HiCrome™ ESBL agar. The findings align with the previous study that reported a similar utility of chromogenic ESBL agar for rapid and accurate detection of ESBL-producing bacteria in clinical and environmental samples (Huang

et al., 2010). Similarly, another study identified clinical *E. coli* isolates as ESBL producers using both chromogenic media indicating the widespread occurrence of ESBL-producing strains (Glupczynski et al., 2007).

Table 1: The table presents the zone of inhibition for antibiotics in the Hexa G-minus 23 antibiotic ring tested against ESBL-positive isolates

HEXA G-minus 23						
	Zone of Inhibition (mm)					
Isolates	**AT**	**CCL**	**CAZ**	**CTX**	**CPD**	**CTR**
1	12	8	-	-	-	-
2	-	-	-	-	-	-
3	11	-	-	-	-	-
4	10	8	-	-	-	-
5	14	-	-	-	-	-
6	8	-	7	-	-	-
7	12	7	-	10	-	-
8	11	-	12	-	-	-
9	11	-	-	-	-	-
10	12	-	12	11	-	-
Control	29	30	25	29	30	28

Furthermore, 10 *E. coli* isolates were identified as ESBL producers based on their reduced Zone of Inhibition (ZoI) against the antibiotics CTX, CTR, CAZ, CPD, CCL, and AT, as per CLSI guidelines. Notably, all 10 isolates demonstrated complete resistance (100%) to CTR and CPD that is consistent with other findings (Ramachandran et al., 2021; Sakai et al., 2023).

The AST conducted through the VITEK-2 system revealed a concerning MDR profile among ESBL-positive *E. coli* isolates. The isolates demonstrated 100% resistance to thirteen out of the thirty antibiotics tested, including commonly used β-lactams, fluoroquinolones, and polymyxins, which account for 43.3% of the antibiotics tested. These findings are consistent with previous studies that reported high levels of resistance to β-lactams, including cefotaxime, ceftazidime, and cefepime, in ESBL-producing *E. coli* isolates (Kayastha et al., 2020; Oteo et al., 2006). The 100% resistance to ampicillin and ampicillin/sulbactam observed in this study shows similar results to a study that highlighted the inefficacy of these antibiotics against ESBL-producing strains due to the hydrolysis of the β-lactam ring by ESBL enzymes (Beshiru et al., 2024). The high resistance rates to ciprofloxacin (80%) and levofloxacin (100%) are particularly alarming, as fluoroquinolones are often used as first-line treatment options (Boyd et al., 2008). In contrast, gentamicin, minocycline, and trimethoprim/sulfamethoxazole exhibited complete sensitivity (100%) in this study (Liu et al., 2011). Interestingly, nalidixic acid and tobramycin showed 70% sensitivity, and fosfomycin demonstrated 60% sensitivity, indicating potential utility in treating specific cases (Gharavi et al., 2021; Moaz et al., 1989). The observed moderate resistance to carbapenems (ertapenem 80%, meropenem 70%) is concerning, as carbapenems are often considered the most effective agents against ESBL-producing bacteria (Elliott et al., 2006; Jacoby et al., 2004; Yasmin et al., 2022).

Table 2: Interpretation of Extended-Spectrum Beta-Lactamase (ESBL) positive *Escherichia coli* isolates based on Minimum Inhibitory Concentration (MIC) breakpoints defined by CLSI and EUCAST, categorizing them as Susceptible (S), Intermediate (I), or Resistant (R) against different antibiotics

S. No.	ANTIBIOTICS	INTERPRETATION									
		1	2	3	4	5	6	7	8	9	10
1	Amikacin	I	R	I	I	R	R	R	I	R	R
2	Amoxicillin/Clavulanate	I	I	I	I	R	I	R	R	R	I
3	Ampicillin	R	R	R	R	R	R	R	R	R	R
4	Ampicillin/Sulbactam	R	R	R	R	R	R	R	R	R	R
5	Cefazolin	R	R	R	R	R	R	R	R	R	R
6	Cefepime	R	R	R	R	R	R	R	R	R	R
7	Cefixime	R	R	R	R	R	R	R	R	R	R
8	Cefotaxime	R	R	R	R	R	R	R	R	R	R
9	Cefoxitin	R	R	R	R	R	R	R	R	R	R
10	Ceftazidime	R	R	R	R	R	R	R	R	R	R
11	Cefuroxime	R	R	R	R	R	R	R	R	R	R
12	Chloramphenicol	R	R	R	R	R	R	R	R	R	R
13	Ciprofloxacin	R	S	R	R	R	R	S	R	R	R
14	Colistin	R	R	R	R	R	R	R	R	R	R
15	Ertapenem	I	R	I	R	R	R	R	R	R	R
16	Fosfomycin	S	R	S	I	I	S	S	S	I	S
17	Gentamicin	S	S	S	S	S	S	S	S	S	S
18	Imipenem	I	I	I	I	I	I	I	I	I	R
19	Levofloxacin	R	R	R	R	R	R	R	R	R	R
20	Meropenem	I	I	I	S	I	I	I	S	I	S
21	Minocycline	S	S	S	S	S	S	S	S	S	S
22	Nalidixic Acid	R	S	S	S	S	R	S	S	S	R
23	Nitrofurantoin	R	R	R	R	R	R	I	R	R	R
24	Norfloxacin	R	R	R	R	R	R	R	R	R	R
25	Piperacillin/Tazobactam	I	I	I	R	R	R	R	R	R	R
26	Polymyxin B	R	R	R	R	R	I	I	R	R	R
27	Tetracycline	S	S	S	R	R	R	R	R	R	R
28	Tigecycline	I	I	S	I	I	S	S	I	I	R
29	Tobramycin	S	S	S	I	S	I	I	S	S	S
30	Trimethoprim/Sulfamethoxazole	S	S	S	S	S	S	S	S	S	S

**R- Resistant, I- Intermediate, S- Sensitivity*

CONCLUSION

This study underscores the significant prevalence of *E. coli* (27.4%) in environmental samples and highlights the robust utility of EMB agar for its effective isolation. Additionally, the identification of

23.25% of these isolates as ESBL producers using HiCrome™ ESBL agar confirms the widespread occurrence of these resistant strains. The alarming resistance profiles revealed by the VITEK-2 system, including 100% resistance to several commonly used antibiotics, emphasize the critical challenge posed by MDR *E. coli*. The high resistance rates to β-lactams and fluoroquinolones, and the moderate resistance to carbapenems, reflect a troubling trend that complicates treatment options. However, the complete sensitivity of isolates to gentamicin, minocycline, and trimethoprim/sulfamethoxazole provides a hope for alternative therapeutic strategies. These findings highlight the urgent need for continuous surveillance, judicious use of antibiotics, and further research into alternative treatment options to effectively manage and mitigate the spread of MDR *E. coli*. Addressing this issue is crucial for public health, requiring coordinated efforts in environmental monitoring, clinical practice, and antibiotic stewardship.

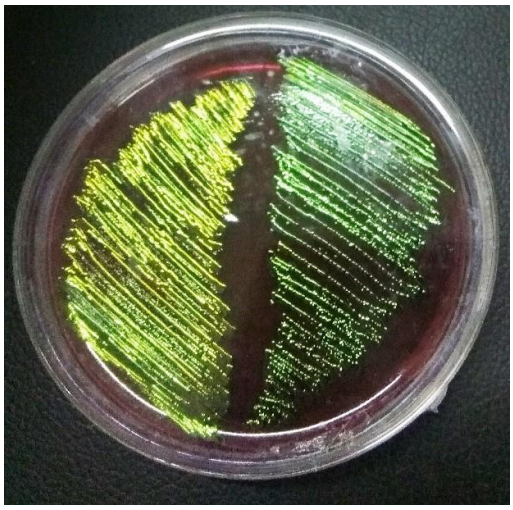

Figure 1: The image shows *Escherichia coli* colonies on Eosin-Methylene Blue (EMB) agar as they produce a characteristic metallic green sheen due to the fermentation of lactose and sucrose leading to acid production, which interacts with the eosin and methylene blue dyes.

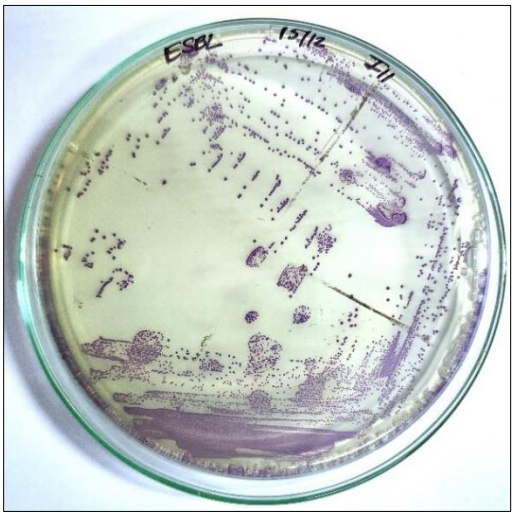

Figure 2: The image depicts the growth of Extended Spectrum Beta-Lactamase (ESBL)-producing *Escherichia coli* on HiCrome™ ESBL agar as distinct purple-coloured colonies

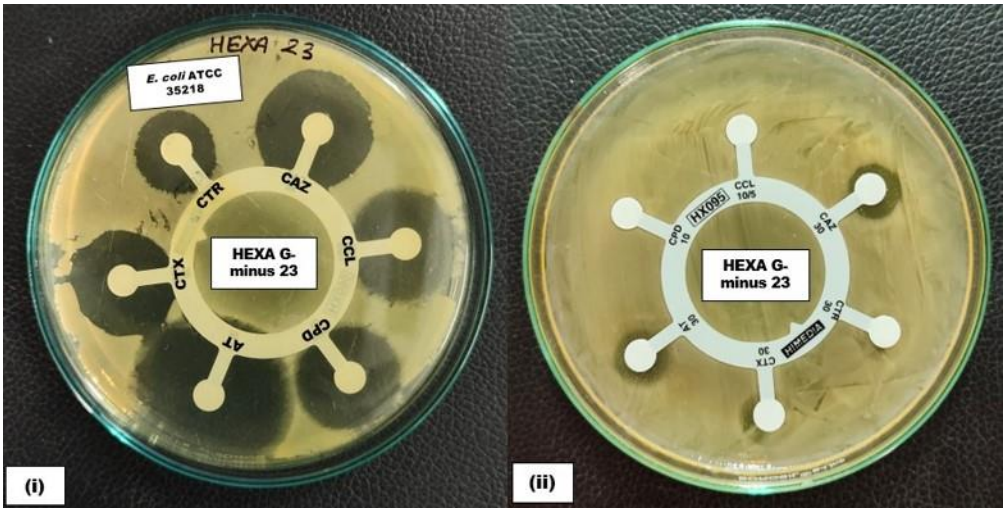

Figure 3: Hexa G-minus antibiotic ring for the detection of ESBL positive *E. coli*: (i) *E. coli* ATCC 35218 displaying susceptibility to all antibiotics in the Hexa G-minus antibiotic ring, characterized by clear zones of inhibition around all antibiotic discs (ii) ESBL positive isolate shows resistance to all antibiotics in the ring, with noticeably smaller zones of inhibition around aztreonam, ceftazidime, and cefotaxime. This resistance pattern is indicative of ESBL production, as these enzymes confer resistance to beta-lactam antibiotics, including third-generation cephalosporins.

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CONFLICT OF INTEREST
On behalf of all authors, the corresponding author states that there is no conflict of interest.

CONSENT STATEMENT
Authors have given their consent to publish the manuscript.

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