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Diagnostic Discordance in ESBL Detection: HiCrome™ Chromogenic Agar and DDST

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ABSTRACT

Background: Extended-spectrum β-lactamases (ESBLs) compromise the efficacy of third-generation cephalosporins and are a major driver of antimicrobial resistance among Enterobacterales. Rapid phenotypic screening (chromogenic agar) and confirmatory phenotypic tests (double-disc synergy test, DDST) are widely used in routine laboratories, but discordance between these methods has diagnostic and therapeutic implications.

Objectives: To evaluate the prevalence of phenotypic ESBL indicators among third-generation cephalosporin-resistant Enterobacterales and to quantify agreement and discordance between HiCrome™ ESBL chromogenic agar screening and DDST confirmation in a tertiary-care centre in Haryana.

Methods: Observational study of 150 Enterobacteralesisolates procured at Microbiology laboratory that were resistant to ceftazidime, cefotaxime or ceftriaxone by CLSI disk diffusion. Chromogenic screening (HiCrome™ ESBL Agar) and DDST were performed on all 150 isolates. Agreement was assessed using Cohen's κ and McNemar's test; stratified analyses and logistic regression explored predictors of discordance. PCR confirmation was planned for dual-positive isolates in a subsequent study.

Results: Of 150 isolates, 119 (79.3%) were chromogenic-positive and 73 (48.7%) were DDST-positive. The 2×2 contingency yielded: both positive = 73; chromogenic+/DDST− = 46; chromogenic−/DDST+ = 0; both negative = 31. Observed agreement = 0.693; expected agreement = 0.491; Cohen's κ = 0.398 (moderate agreement). McNemar's test (continuity corrected) $\chi^2 \approx 44.02$, $p < 0.0001$, indicating significant directional discordance (predominantly chromogenic positives lacking DDST confirmation). Discordance was more frequent among Escherichia coli and urine isolates.

Conclusions: HiCrome™ chromogenic screening yields a high proportion of presumptive ESBL positives but shows only moderate agreement with DDST; many chromogenic positives lack clavulanate synergy. The observed discordance has important implications for antimicrobial stewardship and infection control.

Keywords: *ESBL,Enterobacterales, Chromogenic agar, Double-disc synergy test, Diagnostic agreement, Cohen's kappa,McNemar's test, India.*

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1. INTRODUCTION

The global escalation of antimicrobial resistance (AMR) among Gram-negative bacteria represents one of the most alarming threats to modern medicine1, 2. Among the mechanisms that erode the efficacy of β-lactam antibiotics, extended-spectrum β-lactamases (ESBLs) are particularly consequential: they hydrolyse third-generation cephalosporins and aztreonam, often co-occurring with resistance determinants to other antibiotic classes, thereby narrowing therapeutic options and increasing morbidity, mortality and healthcare costs3, 4. ESBLs are diverse: historically dominated by TEM and SHV families, the CTX-M family has emerged over the last two decades as the most widespread lineage globally, driven by plasmid mobility and clonal expansion5, 6. Other families (OXA variants, PER, VEB, GES) contribute to local

epidemiology and complicate detection strategies7, 8.

Accurate and timely laboratory detection of ESBL production is essential for appropriate antimicrobial therapy, infection control and surveillance9. In many clinical microbiology laboratories, phenotypic methods remain the backbone of ESBL detection because they are cost-effective and technically accessible10. Two broad phenotypic approaches are commonly used: screening methods that rapidly flag potential ESBL producers (chromogenic media designed to indicate ESBL activity by colony colour) and confirmatory methods that demonstrate inhibition of β-lactamase activity by β-lactamase inhibitors (for example, the double-disc synergy test, DDST, or combined disk tests). Chromogenic media such as HiCrome™ ESBL Agar provide a rapid, visually intuitive screening tool that can simultaneously support species presumptive identification and ESBL screening9. DDST, recommended in various forms by clinical guidelines, detects synergy between cephalosporins and clavulanic acid and is widely used as a phenotypic confirmatory test.

However, these methods differ fundamentally in principle and performance. Chromogenic media detect growth patterns and colour changes that correlate with enzyme activity or substrate utilization, while DDST directly assesses inhibitor-mediated restoration of susceptibility9. Consequently, discordance between chromogenic screening and DDST confirmation has been reported in multiple settings. Discordance may arise from several biological and technical factors: co-production of AmpC β-lactamases (which are not inhibited by clavulanate), inhibitor-resistant TEM (IRT) variants, low-level or heterogeneous expression of ESBLs, inoculum effects, and differences in sensitivity and specificity inherent to the assays11, 12. In resource-limited settings, where molecular confirmation (PCR) is not always feasible, understanding the performance and limitations of phenotypic methods is critical for designing pragmatic diagnostic algorithms.

India bears a disproportionate burden of AMR, driven by high antibiotic consumption, over-the-counter availability of antibiotics, variable infection control practices, and dense human and animal populations that facilitate transmission. Regional studies from the Delhi-NCR and other Indian centres have documented high prevalence of ESBL-producing Enterobacterales, with CTX-M dominance and frequent co-resistance to non-β-lactam agents13, 14. These epidemiological realities amplify the need for robust, context-appropriate diagnostic strategies.

This study was conducted in a tertiary-care centre in Haryana to evaluate the prevalence of phenotypic ESBL indicators among Enterobacterales isolates resistant to third-generation cephalosporins and to quantify the agreement and discordance between HiCrome™ chromogenic agar screening and DDST confirmation. The present study focuses on phenotypic comparison and its implications and emphasize the discordance as a clinically relevant and novel observation in this study, the present study also proposes a staged diagnostic approach that reserves molecular confirmation for isolates fulfilling dual-positive phenotypic criteria.

2. MATERIALS AND METHODS

Study design and setting: This observational laboratory study was performed in the Department of Microbiology, SGT Hospital and Research Centre, Faculty of Medicine and Health Sciences, SGT University, Budhera, Gurugram, Haryana, India. The isolates used in this study were received as part of routine diagnostic work from various departments of the hospital including both IPD and OPD.

Sample selection and inclusion criteria: A total of 300Enterobacterales isolates were initially screened as part of routine antimicrobial susceptibility testing (AST). From these, 150 isolates that demonstrated resistance to at least one third-generation cephalosporin—defined as ceftazidime zone diameter <20 mm, cefotaxime <20 mm, or ceftriaxone <20 mm by Kirby-Bauer disk diffusion—were included as potential ESBL producers for further phenotypic evaluation. Non-Enterobacterales isolates and isolates lacking complete AST data were excluded.

Ethical considerations: The study used anonymized clinical isolates collected during routine diagnostic procedures. Institutional Ethics Committee approval was obtained prior to study initiation and a waiver of individual informed consent was granted for the use of de-identified bacterial isolates and associated minimal metadata (age group, sex, specimen type). All laboratory work complied with institutional biosafety policies.

Microbiological identification and routine AST: Isolates were identified to species level using a combination of colony morphology, standard biochemical testsand the Vitek-2 automated identification system (as per the tests prescribed). Antimicrobial susceptibility testing was performed by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA) following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI M100)21. Disks used included ceftazidime (30 µg), cefotaxime (30 µg) and ceftriaxone (30 µg) along with others. Zone diameters were measured and interpreted as per CLSI breakpoints at the time of testing.

Chromogenic agar screening (HiCrome™ ESBL Agar): HiCrome™ ESBL Agar Base (HiMedia Laboratories;

product MB1829) was prepared according to the manufacturer's instructions and supplemented with AC3F selective supplement as recommended. Plates were inoculated with isolates from pure cultures and incubated at 35–37°C for 18–24 hours. Colony colour interpretation followed the manufacturer's guidance22: pink/purple colonies suggest ESBL-producing E. coli; blue-green colonies suggest ESBL-producing Klebsiella spp.; no colour change or scant growth was interpreted as possible weak enzyme expression or non-ESBL.

Double-disc synergy test (DDST) — Phenotypic confirmation: DDST was performed on Mueller-Hinton agar using standard methodology. Briefly, 4–5 colonies from an overnight culture were suspended in sterile normal saline and adjusted to 0.5 McFarland turbidity. Lawn cultures were prepared and allowed to dry for 3–5 minutes. Antibiotic discs were placed on the agar surface: ceftazidime (30 µg) and ceftazidime + clavulanic acid (30 µg + 10 µg), cefotaxime (30 µg) and cefotaxime + clavulanic acid (30 µg + 10 µg). Plates were incubated at 35–37°C for 16–18 hours. An increase of ≥5 mm in the zone diameter for the combination disc compared with the cephalosporin alone was considered confirmatory for ESBL production, consistent with CLSI guidance15-19.

Data collection and variables: For each isolate the following variables were recorded: unique isolate identifier, species, specimen type (urine, blood, pus/exudate, sputum, other), patient sex and age group (1–20, 21–40, 41–60, >61), AST zone diameters for ceftazidime, cefotaxime and ceftriaxone, chromogenic agar result (colour/no growth/luxuriant no colour), DDST result (positive/negative and zone diameters), and dual-positive status (chromogenic+ & DDST+). PCR results were not included in this manuscript; molecular testing of dual-positive isolates is planned and will be reported separately.

Statistical analysis: Data were entered into a spreadsheet and analysed using standard statistical software. Descriptive statistics (counts, percentages, 95% confidence intervals) summarized isolate distribution, specimen types and phenotypic results. Agreement between chromogenic agar and DDST was assessed using Cohen's kappa coefficient (κ) with interpretation following conventional thresholds (κ <0.20 poor; 0.21–0.40 fair; 0.41–0.60 moderate; 0.61–0.80 substantial; >0.80 almost perfect). McNemar's test (continuity corrected) evaluated directional discordance in paired binary outcomes. Stratified analyses (species, specimen type, age group, sex) used chi-square or Fisher's exact tests as appropriate. Logistic regression was used to explore predictors of discordance (outcome: chromogenic+/DDST− vs concordant results)with variables selected based on biological plausibility and univariable screening. A two-tailed p-value <0.05 was considered statistically significant20. Exact calculations for Cohen's κ and McNemar's test are presented in the result.

3. RESULT & DISCUSSION

From the initial pool of Enterobacterales isolates screened in the microbiology laboratory, 150 were identified as resistant to at least one third- generation cephalosporin and therefore selected for detailed phenotypic evaluation. This high level of resistance itself underscores the burden of antimicrobial resistance in the hospital setting. All 150 isolates demonstrated resistance to cefotaxime, nearly all to ceftriaxone (98.7%), and the majority to ceftazidime (96.7%). These figures reflect the widespread compromise of third- generation cephalosporins in routine clinical practice.

Species distribution revealed a clear predominance of Escherichia coli, accounting for nearly two- thirds of the isolates (95/150, 63.3%). Klebsiella pneumoniae was the second most common species (44/150, 29.3%), while other genera such as Acinetobacter, Proteus and others were represented in smaller numbers. This pattern is consistent with the epidemiology of ESBL producers in India, where E. coli and K. pneumoniae dominate both community and hospital infections. The predominance of E. coli also reflects the high proportion of urinary tract infections in the study.

Table 1. Species distribution of*Enterobacterales* (n = 150)

Organism	No. (%)
E. coli	95 (63.3)
K. pneumoniae	44 (29.3)
Acinetobacter spp.	5 (3.3)
Proteus spp.	4 (2.7)
Other species	2 (1.3)

Figure 1. Species distribution of*Enterobacterales* (n = 150)

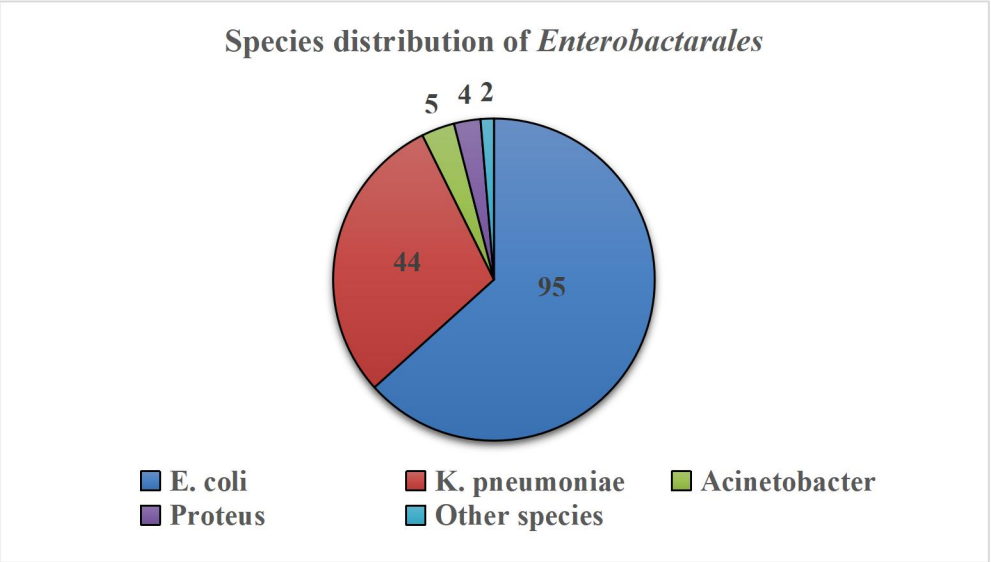
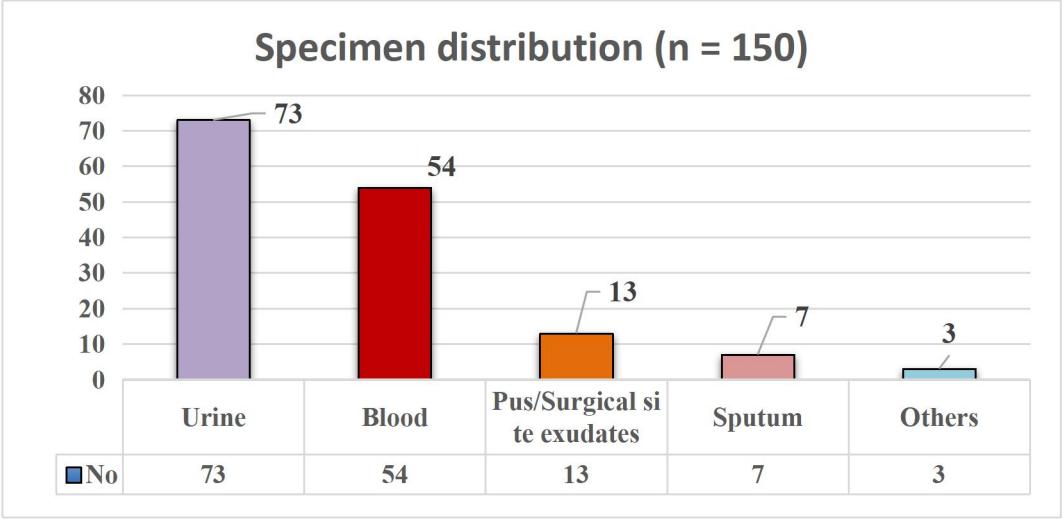


Table 2. Specimen distribution (n = 150)

Specimen	No. (%)
Urine	73 (48.7)
Blood	54 (36.0)
Pus/exudates	13 (8.7)
Sputum	7 (4.7)
Others	3 (2.0)

Figure 2. Specimen distribution (n = 150)



Specimen distribution further supports this observation: urine was the most frequent source (73/150, 48.7%), followed by blood (54/150, 36%). Pus/exudates, sputum, and other specimens contributed smaller proportions. The high number of urinary isolates is in line with the clinical burden of ESBL-producing *E. coli* in urinary tract infections, while the substantial number of bloodstream isolates highlights the serious invasive potential of these organisms.

Demographic analysis showed that females contributed a larger share of isolates (91/150, 60.7%) compared with males (59/150, 39.3%). The age distribution revealed that the 41 – 60years group was most affected (38%), followed by younger adults aged

21–40 years (31.3%). These findings suggest that ESBL infections are not confined to the elderly but affect a wide age range, with a notable burden in middle-aged adults.

Table 3. Age and sex distribution

Parameter	Category	No. (%)
Sex	Male	59 (39.3)
	Female	91 (60.7)
Age	1–20	24 (16.0)
	21–40	47 (31.3)
	41–60	57 (38.0)
	>61	22 (14.7)

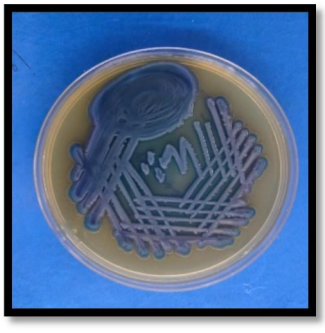
Table 4. Contingency table: Chromogenic agar vs DDST

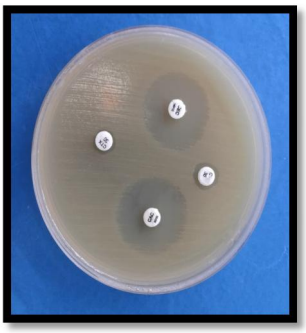
	DDST+	DDST−	Total
ChromAgar+	73	46	119
ChromAgar−	0	31	31
Total	73	77	150

Table 5. Discordant isolates by species

Species	ChromAgar+/DDST−	% of species isolates
E. coli	32	33.7%
K. pneumoniae	10	22.7%
Others	4	–

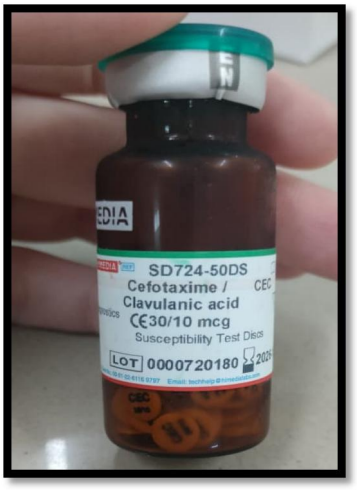
Phenotypic testing produced striking results. Chromogenic agar screening identified 119 isolates (79.3%) as presumptive ESBL producers based on colony colour. In contrast, DDST confirmed ESBL production in only 73 isolates (48.7%). The contingency table illustrates this discordance: while 73 isolates were positive by both methods, 46 were positive on chromogenic agar but negative by DDSTand 31 were negative by both. No isolate was DDST positive without chromogenic positivity. This asymmetry indicates that chromogenic agar tends to over-call ESBL positivity compared with DDST.

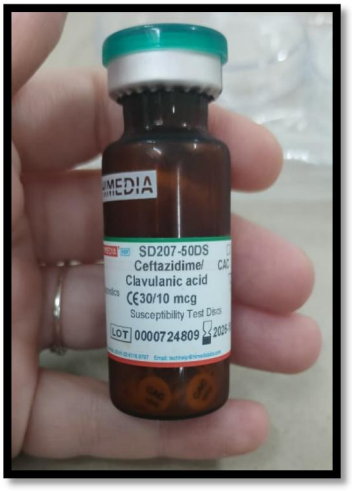




Statistical analysis confirmed these impressions. Overall agreement between the two methods was moderate, with Cohen's kappa around 0.40. McNemar's test demonstrated highly significant discordance ($p < 0.0001$), driven by the large number of chromogenic positives lacking DDST confirmation. Importantly, discordance was not evenly distributed: *E. coli* accounted for the majority of discordant isolates, particularly those from urine specimens. This suggests that biological factors such as AmpC co-production or inhibitor-resistant variants may be more prevalent in *E. coli* urinary isolates, leading to chromogenic positivity without DDST confirmation.

Chromogenic agar interpretations also revealed variability. While most isolates produced characteristic colours, 22 showed luxuriant growth without colour change and nine showed no growth at all, despite being resistant to third- generation cephalosporins. These findings highlight limitations of chromogenic media, which may fail to detect certain phenotypes or produce ambiguous results.





Taken together, the results demonstrate a high prevalence of presumptive ESBL producers among cephalosporin- resistant *Enterobacterales*, but also a significant discordance between screening and confirmatory phenotypic methods. The predominance of *E. coli* and urinary isolates among discordant cases emphasizes the need for careful interpretation and the importance of confirmatory testing.

4. DISCUSSION

The present study highlights a substantial burden of phenotypic ESBL indicators among third-generation cephalosporin-resistant *Enterobacterales*, alongside a pronounced discordance between chromogenic agar screening and DDST confirmation. This finding reflects the growing complexity of ESBL detection in routine microbiology laboratories and underscores the limitations of relying on single phenotypic methods for clinical decision-making.

The predominance of *Escherichia coli* and *Klebsiella pneumoniae* observed in this study is consistent with global and regional epidemiological trends, where these organisms are recognized as the principal reservoirs of ESBL genes, particularly CTX-M variants[4,6]. The widespread dissemination of CTX-M enzymes has been attributed to plasmid-mediated horizontal gene transfer and clonal expansion, facilitating their rapid spread across both community and healthcare settings[6]. Similar studies have reported that E. coli remains the most frequent ESBL producer, especially in urinary isolates, which aligns with the high proportion of urine samples observed in the present study.The high prevalence of resistance to third-generation cephalosporins observed in this study reflects the extensive selective pressure exerted by antibiotic use in hospital and community settings. ESBL-producing organisms are frequently associated with multidrug resistance, limiting therapeutic options and increasing reliance on last-line agents such as carbapenems[12]. This trend is particularly concerning in developing countries, including India, where antimicrobial stewardship practices may be inconsistently implemented[20].A key finding of this study is the marked discordance between chromogenic agar and DDST results. Chromogenic agar identified a substantially higher number of presumptive ESBL producers compared with DDST, indicating higher sensitivity but lower specificity. This observation is consistent with previous studies demonstrating that chromogenic media are highly sensitive screening tools but may generate false-positive results due to detection of non-ESBL resistance mechanisms[22,23]. Comparative evaluations have shown that chromogenic agar can achieve sensitivity approaching 98–99%, whereas DDST, although specific, may fail to detect certain ESBL variants.

The asymmetrical discordance pattern observed in this study—where chromogenic-positive/DDST-negative isolates predominated—has important biological explanations. One of the most significant contributors is the co-production of AmpC β-lactamases, which hydrolyse cephalosporins but are not inhibited by clavulanic acid. As a result, isolates producing both ESBL and AmpC enzymes may appear positive on chromogenic media but fail to demonstrate synergy in DDST, leading to false-negative confirmatory results[24]. Evidence suggests that AmpC co-production can significantly reduce the sensitivity of DDST, particularly in organisms such as *E. coli* and *K. pneumoniae.*

In addition to AmpC, inhibitor-resistant TEM (IRT) and SHV variants may contribute to discordance by reducing the effectiveness of clavulanate-based detection methods[25]. Low-level or heterogeneous expression of ESBL genes may also produce detectable activity on chromogenic substrates without generating sufficient zone enhancement in DDST. Furthermore, technical variables—including inoculum density, disc spacing, incubation conditions, and subjective interpretation—can influence test outcomes and contribute to variability between methods[10].The predominance of discordant isolates among *E. coli*, particularly from urine samples, is a notable finding. This observation is supported by previous studies, which have reported higher rates of discordance in urinary isolates due to the increased prevalence of plasmid-mediated AmpC and mixed resistance mechanisms in these strains[12]. The urinary tract environment, coupled with repeated antibiotic exposure, may further select for complex resistance phenotypes, thereby complicating phenotypic detection.

From a clinical perspective, the implications of such discordance are significant. Overestimation of ESBL prevalence based on chromogenic screening alone may lead to unnecessary escalation of therapy to carbapenems, thereby accelerating the emergence of carbapenem resistance. Conversely, reliance solely on DDST may result in under-detection of ESBL producers, leading to inappropriate use of cephalosporins and potential treatment failure[26]. This diagnostic uncertainty directly impacts antimicrobial stewardship efforts and highlights the need for balanced, evidence-based laboratory algorithms.

The findings of this study support the adoption of a staged diagnostic approach for ESBL detection. Initial screening using chromogenic media can provide rapid identification of potential ESBL producers, while confirmatory testing using DDST improves specificity. Molecular methods such as PCR remain the gold standard for definitive identification but are often limited by cost and infrastructure constraints in resource-limited settings. Therefore, reserving molecular testing for isolates that are positive by both phenotypic methods represents a pragmatic and cost-effective strategy.In the broader context of infection control and surveillance, the observed discordance emphasizes the need for standardized laboratory protocols and quality assurance measures. Laboratories should report ESBL detection results with clear indication of the methods used and acknowledge the potential for discordance. Integration of phenotypic and molecular data in surveillance programs is essential for accurate estimation of ESBL prevalence and for tracking the spread of specific resistance genes.

Overall, this study demonstrates that phenotypic discordance is not an anomaly but a common and clinically relevant phenomenon in ESBL detection. Chromogenic agar serves as a sensitive screening tool but tends to overestimate ESBL prevalence, whereas DDST provides specificity but may miss certain resistant phenotypes. The combined use of these methods, supported by molecular confirmation where feasible, offers the most reliable strategy for accurate detection and optimal patient management.

Limitations of the study: The present study is single-centre and may not be generalizable to other regions of India. The inclusion criterion (resistance to third-generation cephalosporins) enriches for resistant isolates and may overestimate phenotypic ESBL prevalence compared with unselected clinical isolates. Molecular confirmation (PCR) was not performed for the isolates in this present study. The study did not perform specific tests for AmpC or IRT variants, which limits mechanistic attribution of discordance.

5. CONCLUSION

In this tertiary-care studyfrom Haryana, HiCrome™ chromogenic agar screening identified a large pool of presumptive ESBL producers among third-generation cephalosporin-resistant *Enterobacterales*, but only about half of these were confirmed by DDST. The moderate Cohen's κ and highly significant McNemar's test indicate substantial and directional discordance, primarily chromogenic positives lacking clavulanate synergy. These findings underscore the limitations of relying on a single phenotypic method and support a staged diagnostic approach that reserves molecular confirmation for isolates meeting dual-positive phenotypic criteria. Given the high burden of ESBLs in India and the clinical consequences of misclassification, laboratories should adopt combined phenotypic strategies and pursue molecular characterization to guide therapy and infection control.

DECLARATIONS

Conflicts of interest: There is no any conflict of interest associated with this study

Consent to participate: There is consent to participate.

Consent for publication: There is consent for the publication of this paper.

Authors' contributions: Author equally contributed the work.

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