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Exploring New Delhi Metallo Beta Lactamases in *Klebsiella pneumoniae* and *Escherichia coli*: genotypic vs. phenotypic insights

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Abstract

Background Carbapenemase-producing *Enterobacterales* pose a serious clinical threat, particularly in high-burden settings of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* (CREK), where rapid detection tools are essential to aid patient management. In this study, we focused on bla_{NDM}, the most frequently reported carbapenemase in the region, and evaluated a combined phenotypic (lateral flow) and genotypic (PCR and WGS) approach for its detection. This research underscores the utility of lateral flow assays as a practical alternative to resource-intensive genotypic methods, offering a scalable solution for settings with limited laboratory capacity.

Method One hundred seventy-seven extensively drug-resistant strains were characterized using MALDI-TOF. Isolates were analyzed to detect Carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* (CREK) using disk diffusion, MIC test, and PCR targeting bla_{NDM}. Antibiotic susceptibility patterns were analyzed and visualized using single-linkage hierarchical clustering, with results displayed on a permuted heat map. Immunochromatographic assay, RESIST-5 O.K.N.V.I (Coris Bioconcept®) was used for CREK isolates [($n=17$), positive and negative)] and Oxford Nanopore Sequencing was conducted on subsets [($n=5$) bla_{NDM}-positive co-producers of bla_{NDM} and bla_{OXA}, and ($n=2$) bla_{NDM}-negative bla_{OXA} producers) to evaluate the reliability of phenotypic and genotypic tests.

Result Most of the XDR strains (90%) were CREK, with *K. pneumoniae* (71.2%) more prevalent than *E. coli* (28.7%) ($p<0.05$). All CREK strains exhibited complete resistance (100%) to multiple antibiotics with 66% showing sensitivity to levofloxacin. Furthermore, *K. pneumoniae* (57.8%) had higher bla_{NDM} gene prevalence than *E. coli* (36.9%). Among bla_{NDM}-positive CREK, lateral flow assay revealed approximately half of each bacteria type co-produced bla_{OXA} (*E. coli*, 52.9%), and (*K. pneumoniae*, 47%). For bla_{NDM}-negative strains, bla_{OXA} was more prevalent in *K. pneumoniae* (82.35%) than *E. coli* (41%) ($p<0.05$). Comparing phenotypic to genotypic assays, *E. coli* showed 100% (CI 80.49 – 100%) sensitivity and specificity with a high Kappa agreement coefficient (0.91) (CI 95% 0.661–1, $p<0.01$), whereas *K. pneumoniae* assays had lower sensitivity and specificity (40%) (CI 5.27 – 85.34%), with a lower Kappa agreement coefficient (0.20) (CI 95% 0.104–0.298, $p<0.01$).

Conclusion This study demonstrates the value of the RESIST-5 O.K.N.V.I. lateral flow assay as a rapid and reliable diagnostic tool for detecting bla_{NDM} in *Escherichia coli*, with strong agreement to PCR and WGS. While performance

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for *Klebsiella pneumoniae* was lower, the assay offers a practical alternative in resource-limited settings, aiding antimicrobial stewardship and improving diagnostic capacities in high-burden regions.

Keywords Antimicrobial resistance, Carbapenem resistance, Carbapenemases extensive drug resistance, Lateral flow assay, Genotypic, Phenotypic resistance

Introduction

Carbapenem-resistant *E. coli* and *Klebsiella* (CREK) isolates are found worldwide, primarily due to transmission of carbapenemase-encoding genes conferring resistance to carbapenem drugs [1–3]. CREK present substantial clinical and public health concerns as they can spread within healthcare settings and the community [3–5]. This dissemination is alarming due to the limited treatment options available and the associated increase in mortality rates [6, 7].

K. pneumoniae and E.coli are major reservoir for carbapenemases. Five primary carbapenemase-associated genes of concern among CREK are $bla_{\text{OXA-48}}$, bla_{KPC}, bla_{VIM}, bla_{IMP} and bla_{NDM}, whose prevalence and distribution differ by geographic region [8, 9]. Compared to USA China and Europe were KPC enzymes are the frequently reported among CREK, the literature reports bla_{OXA} and bla_{NDM} gene variants to be the most prevalent among CREK in Pakistan [3, 10]. According to a meta-analysis, bla_{NDM} was identified as the most prevalent carbapenemase in 5 out of 9 studies [11], with a recent report indicating its incidence among carbapenem-resistant isolates to be 53.25% [12]. The spread of New Delhi Metallo Beta Lactamases (NDM) is critical to manage, particularly in Pakistan, given its proximity to India, which is considered an epicenter for the emergence and spread of bla_{NDM} [13, 14].

Identifying carbapenemase-producing CREK is critical in the clinical microbiology laboratory [15–18]. This identification holds significant implications for infection control, the selection of appropriate therapy, of establishing surveillance protocols within the hospital setting [19]. Clinical laboratories may employ several diagnostic tools, phenotypic approaches include culture-based techniques, immuno-chromatographic assays (lateral flow kits,) and genotypic methods use gene amplification [20]. These tools enable accurate detection and characterization of CREK, supporting effective management of infections and implementation of control measures in healthcare settings [21–23].

In resource-deficient settings such as lower middle-income countries (LMICs), culture-based assays are preferred due to cost constraints [24–26]. However, their extended turnaround times for diagnosis make them less suitable for efficient and timely reporting of results than molecular or genotypic methods [27, 28]. However, these methods require specialized expertise and equipment, yet they offer the advantage of being faster and more accurate [18]. Additionally, whole genome sequencing (WGS) delivers detailed insights into the underlying resistome and virulome mechanisms, which can be effective for controlling the dissemination of carbapenem-resistant (CR) and ensuring accurate diagnosis of CREK pathogens [29, 30]. However, its implementation is hindered by the need for expensive equipment and specialized expertise, posing challenges in resource-limited settings, particularly in developing countries [31–33].

Keeping in view the urgency of the issue and making possible the timely treatment of patients suffering from deadly infections, various Lateral flow kits, such as the RESIST-5 O.K.N.V.I used here, are available commercially, which provide rapid detection and characterization of the carbapenemase-resistant organisms with good sensitivity (99.4–100%) and specificity (100%) [34–37]. Nevertheless, there is a risk of cross-reactivity associated with such assays, primarily due to reliance on antigen-antibody interactions, especially when dealing with isolates containing bla_{OXA}, although there have been no reported instances for bla_{NDM} [38]. Therefore, accurate results from these assays are important for effective management of the infection, prevention and dissemination of CREK.

Our objective was to evaluate the effectiveness of the RESIST-5 O.K.N.V.I (CORIS Bio Concept, Gembloux, Belgium) lateral flow immunochromatographic assay kit in conjunction with PCR and WGS for identification of carbapenemases, with a specific emphasis on detecting bla_{NDM} types within CREK strains.

Material and methods study design

This seven-year study (2015–2021) was conducted in two main phases. Initially, at the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan and CitiLab and Research Center, Lahore Pakistan where samples underwent preliminary identification and molecular screening for CR. Subsequently, advanced genomic studies, including Oxford Nanopore sequencing, were carried out at the Center of Clinical Microbiology, University College London.

The bacterial isolates were collected from three major government hospitals and one referral diagnostic center. The bacterial isolates were collected after the ethical approval and consent. The hospitals'

primary catchment area is Lahore, which has a population of 6.7 million in the Punjab province of Pakistan. The study employed a comparative design to assess the phenotypic-genotypic detection of the carbapenemase gene, specifically bla_{NDM}, in extensive drug-resistant (XDR) CREK [10].

Bacterial strains

To determine the prevalence of *E. coli* and *K. pneumoniae* among clinical XDR, specimens from patients ($n = 177$) were streaked onto selective agar plates, specifically MacConkey agar and Blood Agar (Oxoid, Basingstoke Hampshire, UK). These agar plates were incubated aerobically at 37°C for 24 h.

All suspected *E. coli* and *K. pneumoniae* isolates from these plates underwent Gram staining (ThermoFisher Scientific, Heysham, UK), were tested for oxidase (Oxoid, Basingstoke, Hampshire, UK), and subjected to the API 20E system (bioMerieux, Marcy L'Etoile, France) for preliminary identification of *E. coli* and *K. pneumoniae*. Further confirmation was conducted using a MALDI-TOF (MALDI Biotyper, Bruker Daltonics, Billerica, USA) [39, 40].

Antibiogram assay

To assess the antibiotic susceptibility pattern, all isolates were subjected to antibiotic sensitivity testing (AST) using the Kirby-Bauer method on Mueller-Hinton agar (Oxoid, Basingstoke, Hampshire, UK) plates following the Clinical Laboratory Standards Institute (CLSI) recommendations 2017–2019 [41, 42]. The antibiotic panel used for *E. coli* and *K. pneumoniae* included; penicillin's and combinations (amoxicillin 30 µg, amoxicillin-clavulanic acid 40 µg, cefoperazone/Sulbactam 40 µg and piperacillin-tazobactam 30 µg), monobactam (aztreonam 30 µg), extended-spectrum cephalosporins (ceftriaxone 30 µg, cefepime 30 µg, cefotaxime 30 µg, cefoxitin 30 µg, and ceftazidime 30 µg), carbapenems (imipenem 10 µg, meropenem 10 µg), aminoglycosides (amikacin 30 µg and gentamicin 30 µg), a quinolone (ciprofloxacin 30 µg, moxifloxacin 30 µg), folate pathway inhibitors (trimethoprim/Sulphametoxazole). All antibiotic discs were obtained from Oxoid (Basingstoke, Hampshire, UK).

Minimum inhibitory concentration (MICs)

Furthermore, to enhance our understanding of the antimicrobial susceptibility profiles of CREK bacterial strains, we perform a minimum inhibitory concentration (MIC) assay using the agar dilution method for key antimicrobial agents, i.e., imipenem, meropenem, doxycycline, gentamycin, colistin and tigecycline (Oxoid, Basingstoke, Hampshire, UK). The assay was performed using the Mueller Hinton agar plates (Oxoid, Basingstoke, Hampshire, UK) diluted with the appropriate concentration of antibiotics and spotting the prepared inoculum of the bacterial isolates following the already described protocol [43]. The MIC was calculated according to the breakpoints provided by CLSI guidelines.

Detection of New Delhi Metallo Beta Lactamases (bla_{NDM}) gene

To assess the prevalence of the carbapenemase-encoding gene (bla_{NDM}) among CREK, the isolates were further examined for the presence of the bla_{NDM} gene using singleplex PCR. The DNA template used for the PCR was extracted using previously described methods [44]. The PCR reaction was set up with a 25 µL mixture containing 10X PCR buffer, 2.5 mM dNTPs, 20 pmol each primer and 2.5 U of Taq polymerase (Invitrogen, Carlsbad, California, USA). The PCR conditions were set with an initial denaturation at 95 °C. This was followed by 35 cycles of 1 min (min) denaturation at 95 °C, 1.5 min annealing at 53°C, extension for 1 min and final extension for 10 min at 72 °C. The Mg concentration was maintained between 1 and 1.5 mM. The set of primers (F5'ATGGAATTGCCCAATATTATG3', R5'TCAGCGCAGCTTGTCGGCC3') was used to amplify the full-length amplicon of bla_{NDM} gene [45]. Furthermore, positive controls for bla_{NDM} detection in *K. pneumoniae* (ATCC BAA1705) and *E. coli* (ATCC BAA2452) were included.

Lateral flow assay

To detect carbapenemase genes other than bla_{NDM}, we selected bla_{NDM}-positive ($n = 17$) and bla_{NDM}-negative ($n = 17$) isolates from *E. coli* and *K. pneumoniae* strains. These isolates were then tested using the RESIST-5 immunochromatographic lateral flow assay kit with two cassettes (CORIS Bio Concept, Gembloux, Belgium), following the manufacturer's instructions.

The first cassette detects bla_{VIM} and bla_{IMP}, while the second targets bla_{OXA-48}-like variants, bla_{KPC}, and bla_{NDM}, enabling the identification of five major carbapenemase encoding genes. These cassettes feature nitrocellulose membranes sensitised with monoclonal antibodies and employ colloidal gold nanoparticles to target or detect bla_{VIM}, bla_{IMP}, bla_{KPC}, bla_{NDM} and bla_{OXA-48}-like variants.

Whole genome sequencing (WGS)

To accurately identify bla_{NDM} and bla_{OXA} encoding carbapenemase genes and to compare the phenotypic with genotypic detection of bla_{NDM} among CREK strains. We conducted high throughput Oxford Nanopore sequencing on subsets of selected CREK strains: those positive for bla_{NDM} ($n = 5$), co-producers of

bla_{NDM} and bla_{OXA} ($n = 5$), those negative for bla_{NDM} and bla_{OXA} ($n = 2$), and producers of $bla_{OXA\ XA}$ without bla_{NDM} ($n = 2$).

A DNA library was prepared using the ONT Rapid Barcoding 96 Kit (SQK-RBK110.96) (Oxford NANOPORE technologies, Oxford, UK), following the manufacturer's guidelines [46]. Twenty barcoded isolates were run on an R9.4.1 flow cell (ONT) using the Mk1B device for 48 h, using the default parameters of MinKNOW (v21.11.7) software. Base calling was performed using Guppy (v6.0.6) and the flip-flop fast algorithm.

Resistome analysis and plasmid profiling

The drug resistance genes (resistome) were investigated using the Comprehensive Antibiotic Resistance Database (CARD, version 4.0, (https://card.mcmaster.ca/analyze). Briefly, the whole-genome sequence of each isolate was analyzed using the Resistance Gene Identifier (RGI, version 5.2.0) in the Comprehensive Antibiotic Resistance Database (CARD). We employed the parameters 'Perfect and strict hits only', 'Include nudge' and 'high-quality coverage', to predict the antimicrobial resistance genes (ARGs) [47].

Furthermore, plasmid replicon types were determined using Plasmid Finder, version 2.1 of the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org) [48].

Statistical analysis

Statistical computations were performed using SPSS (IBM Corp. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). The demographic distribution across age, gender, department, and specimens was evaluated using the Chi-squared test and one-way ANOVA. Post-hoc pairwise comparisons between groups were conducted through Tukey's multiple-comparison test, with significance at $p < 0.05$. The distribution frequency of the bla_{NDM} gene was calculated as a percentage and subjected to Student t-tests, where statistical significance was defined as $p < 0.05$.

In addition, an analysis of antibiotic susceptibility patterns involved clustering with proximity enhancement, followed by the display of permuted data in a heat map. A hierarchical clustering heat map of antibiotic resistance profiles for isolates was generated using XLSTAT (Addinsoft, New York, USA).

Furthermore, measures of central tendency were computed, and the detection capacity was assessed using the Kappa coefficient between genotypic methods (PCR and WGS) and phenotypic assays (lateral flow). Comparable agreements closer to 1 were considered, and statistical significance was set at $p < 0.05$. Sensitivity %, specificity %, negative predictive (NPV), and positive predictive values (PPV) were calculated for phenotypic and genotypic assays in detecting bla_{NDM} gene among CREK.

Results

Isolation, identification, antibiotic susceptibility pattern and minimum inhibitory concentrations of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* from clinical specimens

Of the 177 XDR isolates, 90.3% (160/177) were confirmed CREK. Within this group, *K. pneumoniae* represented 71.2% (114/160) of the isolates, demonstrating a significantly higher prevalence of carbapenem resistance compared to *E. coli*, which accounted for 28.7% (46/160) of the isolates ($p < 0.05$). Notably, the demographic analysis shows that the majority of *K. pneumoniae* isolates were obtained from urine samples (33.3%, $n = 38/114$) followed by blood specimens (13.1%, $n = 15/114$) among in-patients ($p < 0.05$). Similar patterns were observed for *E. coli* (urine, 41.3%, $n = 19/46$ and blood, 10.8%, $n = 15/46$) (Table 1). Moreover, the disk diffusion method was used to determine the antibiotic susceptibility and similarity pattern between CREK isolates, and antibiotic resistance profiles were analyzed through hierarchical clustering. The CREK isolates exhibited complete resistance to several antibiotics, including ampicillin (100%), Cefoparazone (100%), Ceftriaxone (100%), Cefepime (100%), aztreonam (100%), tetracycline (100%) and augmented (100%). Conversely, the antibiotics with the greatest number of sensitive CREK isolates were tigecycline (23.9% *E. coli* and 6.1% in *K. pneumoniae*) and levofloxacin (66% *E.coli* and 65% in *K. pneumoniae*) (Fig. 1). Hierarchical clustering revealed three primary clusters: A, B, and C. Cluster A grouped four CREK isolates, consisting of two from *E. coli* (E128 and E144) and two from *K. pneumoniae* (K76 and K92). K76 and K92 demonstrated the highest antibiotic susceptibility similarities within cluster A. Cluster B encompassed one hundred and forty-six CREK isolates from *E. coli* (E115 to E122, E124 to E127, E133, E135 to E143, E145 to E153, and E155 to E166) and *K. pneumoniae* (K1 to K5, K8 to K10, K12 to K75, K77 to K91, and K93 to K114). Amongst the *E. coli* isolates in cluster B, there was a notable absence of variation in antibiotic susceptibility similarity. Conversely, *K. pneumoniae* isolates (K3 and K4) and (K35 and K54) displayed no similar antibiotic susceptibility within the same cluster. Additionally, cluster C comprised ten CREK isolates, including seven from *E. coli* (E123, E129 to E132, E134, and E154) and three from *K. pneumoniae* (K6, K7, and K11). All these isolates exhibited the highest similarity in antibiotic susceptibility patterns within cluster C (Fig. 2). Overall, we found that CREK isolates

Table 1 Demographic attributes of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* (CREK) isolated from both in-patients and out-patients

Items	Extensive drug carbapenem-resistant isolates (*n* = numbers)		chi-square statistic	*p*-value
	Escherichia coli	*Klebsiella pneumoniae*		
Age:				
Children	03 (3.16) [0.01]	08 (7.84) [0.00]		
Adolescents	01 (1.44) [0.13]	04 (3.56) [0.05]	9.9743	0.01*
Adults	36 (27.60) [2.56]	60 (68.40) [1.03]		
Older adults	06 (13.80) [4.41]	42 (34.20) [1.78]		
Sex:				
Female	20 (23.29) [0.46]	61 (57.71) [0.19]	1.3192	0.25NS
Male	26 (22.71) [0.48]	53 (56.29) [0.19]		
Department:				
IPD	36 (39.96) [0.39]	103 (99.04) [0.16]	4.2015	0.04*
OPD	10 (6.04) [2.60]	011 (14.96) [1.05]		
Specimens:				
Blood	15 (14.38) [0.03]	35 (35.62) [0.01]		
Catheters	03 (2.88) [0.01]	07 (7.12) [0.00]		
Tracheal secretions	08 (5.75) [0.88]	12 (14.25) [0.36]	4.1006	0.39NS
Urine	19 (19.26) [0.00]	48 (47.74) [0.00]		
Wound and pus	01 (3.74) [2.01]	12 (9.26) [0.81]		

The isolates from both in-patients and out-patients underwent demographic characterization based on Age (following National Institute of Health (NIH) criteria), gender, department, and specimen. Subsequently, statistical analysis was performed on the obtained data.

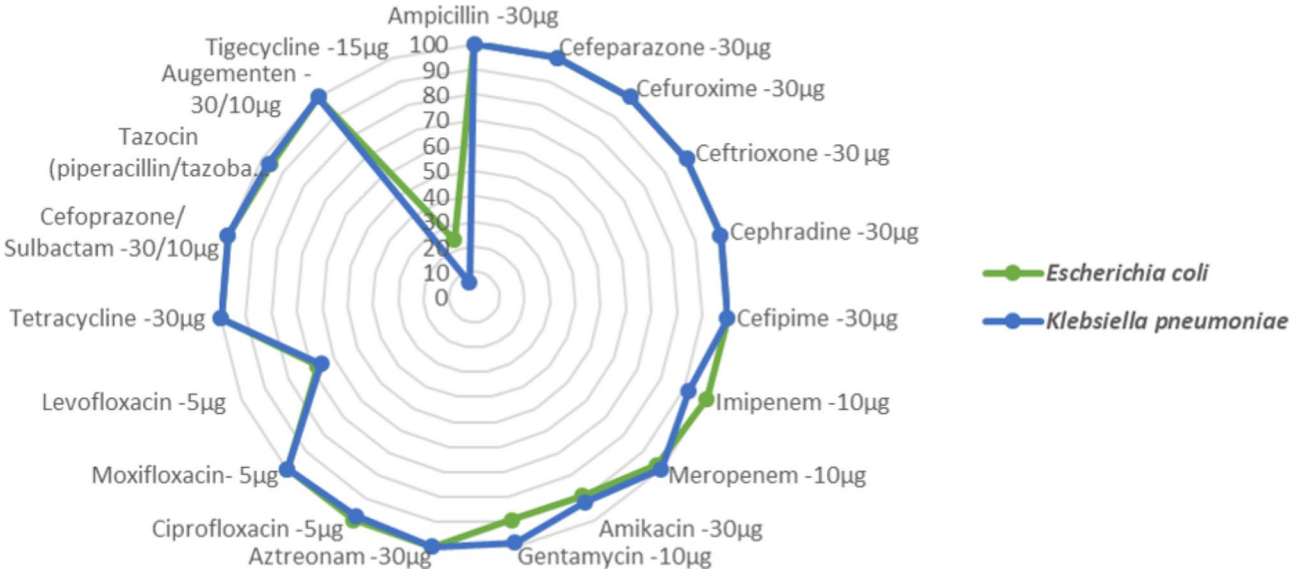

Fig. 1 Antibiotic susceptibility profiling of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* (CREK) isolates. Recommended panel of antibiotics was tested against the CREK isolates to access the overall resistant pattern against the used antibiotics. Results have been presented in the radar graph in terms of percentage calculated as (number of resistant isolates to respective antibiotic / total no of isolates × 100)

demonstrated high rates of sensitivity to colistin, 82.6% for *E. coli* and 87.7% for *K. pneumoniae*, while there were notable rates of sensitivity to tigecycline, 65.2% for *E. coli* and 68.4% for *K. pneumoniae*(***p* < 0.05**). Conversely, the highest rates of resistance were found to be against imipenem (80.7%) and meropenem (100%), characterized by higher breakpoint values (≥ 16 µg/ml), particularly among *K. pneumoniae*, in comparison to *E. coli* (imipenem 76%, meropenem 100% at 16 µg/ml).

Notably, our findings indicated higher rates of resistance to colistin among *E. coli* (13%) compared to *K. pneumoniae* (9.6%), with a similar trend observed for tigecycline, where resistance rates were higher in *E. coli* (28.2%) compared to *K. pneumoniae* (14%) (***p* < 0.05)** (Table 2).

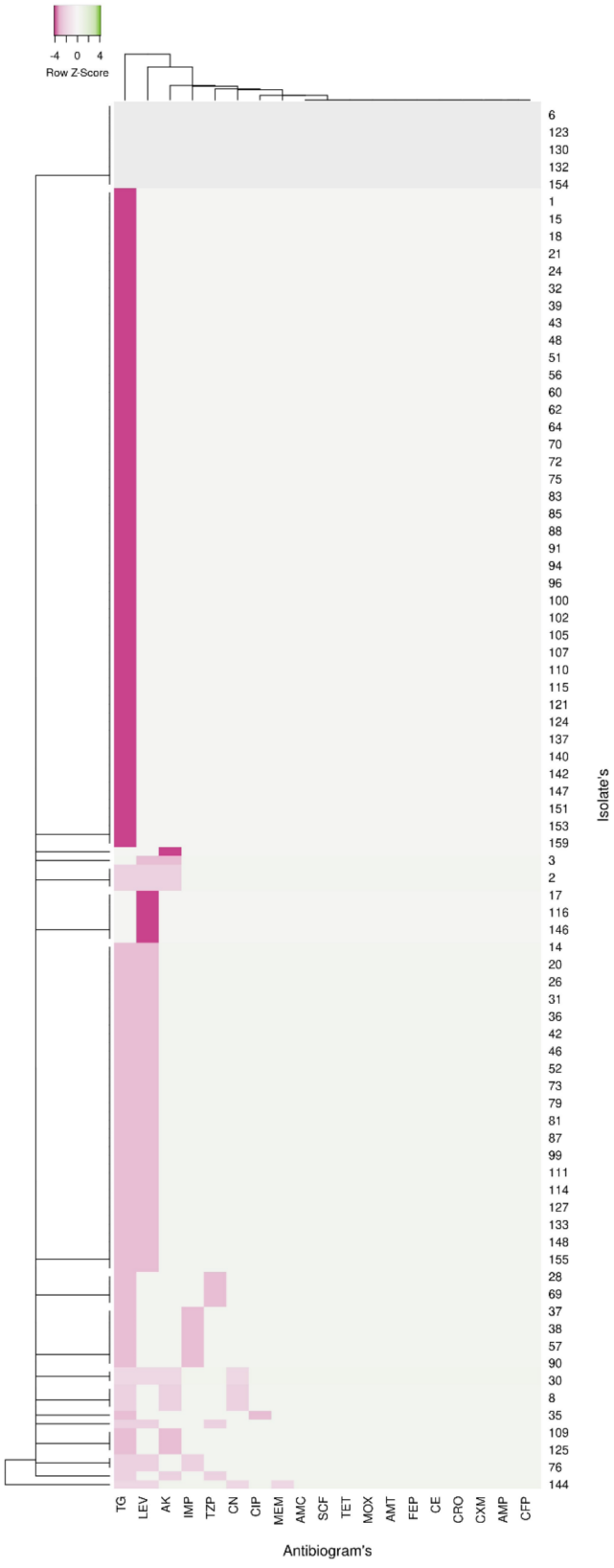

Fig. 2 Heatmap showing hierarchical clustering of isolates antibiotic resistance profiles. Antibiotic susceptibility and similarity pattern between CREK bacterial strains were examined through single linkage clustering, and a heat map was generated using XLSTAT. The antimicrobial resistance profiles are placed horizontally, and CREK strains vertically and phylogenies construct accordingly. Key: CREK, carbapenem-resistant *E. coli* and *K. pneumoniae*

Table 2 Minimum inhibitory concentrations (MIC)

CREK	MIC µg/ ml	Imipenem	Meropenem	Doxycycline	Gentamycin	Colistin	Tigecycline
Escherichia coli	2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	38(82.6%)	30(65.2%)
	4	2 (4.3%)	0 (0%)	0 (0%)	5 (10.8%)	2 (4.3%)	3 (6.5%)
	8	35 (76%)	0 (0%)	0 (0%)	1 (2.1%)	6 (13.0%)	13(28.2%)
	16	5 (10.8%)	46 (100%)	20 (43.4%)	40 (86.9%)	0 (0%)	0 (0%)
	32	1 (2.1%)	0 (0%)	26 (56.52%)	0 (0%)	0 (0%)	0 (0%)
	64	3 (6.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Klebsiella pneumoniae	2	1 (0.8%)	(0%)	0 (0%)	10 (8.7%)	100(87.7%)	78 (68.4%)
	4	4 (3.5%)	(0%)	3 (2.63%)	1 (0.87%)	3 (2.6%)	16 (14.0%)
	8	3 (2.6%)	(0%)	3 (2.63%)	2 (1.75%)	11 (9.6%)	16 (14.0%)
	16	92(80.7%)	114(100%)	108(94.7%)	101(88.5%)	0 (0%)	4 (3.5%)
	32	1 (0.8%)	(0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	64	13 (11.4%	(0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

The minimum inhibitory concentrations were determined using the agar diffusion method following CLSI guidelines. Breakpoint values against *Escherichia coli* and *Klebsiella pneumoniae* were as follows: imipenem and meropenem, sensitive (≤ 1), intermediate (2), and resistant (≥ 4); doxycycline and gentamicin, sensitive (≤ 4), intermediate (8), and resistant (≥ 16). Colistin was classified as intermediate at ≤ 2 and resistant at ≥ 4, while tigecycline was considered resistant at ≥ 2. Key; CREK, carbapenem-resistant *E. coli* and *K. pneumoniae* and MIC, Minimum inhibitory concentration.

Table 3 Distribution patterns of carbapenemase genes were identified using lateral flow assay and precision evaluation of phenotypic test (lateral flow) compared to PCR

Carbapenem resistant bacterial strains	Genotypic method (PCR)	Immunochromatographic method (Lateral flow)					Specificity %	Sensitivity %	CI 95%
		bla_{NDM}	bla_{OXA}	bla_{VIM}	bla_{IMP}	bla_{KPC}			
E. coli	bla_{NDM} negative ($n=17$)	Not detected	7 (41%)	Not detected			100	100	[80.49 – 100%]
	bla_{NDM} Positive ($n=17$)	17 (100%)	9 (52.9%)	Not detected			100	100	
K. pneumoniae	bla_{NDM} negative ($n=17$)	Not detected	14 (82.3%)	Not detected			100	100	
	bla_{NDM} Positive ($n=17$)	17(100%)	8 (47%)	Not detected			100	100	

Distribution frequency of new Delhi Metallo beta-lactamases (bla_{NDM}) gene among carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* using singleplex PCR

The amplified bla_{NDM} gene, yielding a product size of 931 bp was identified in the positive isolates (Supplementary Fig. 1). We found a higher prevalence of bla_{NDM} gene occurrence among *K. pneumoniae*, 57.8% ($n=66/114$), when compared to *E. coli*, 36.9% ($n=17/46$) ($p<0.05$). However, these genotypic results did not agree with the results of phenotypic detection of carbapenem resistance among CREK.

Distribution of carbapenemase genes among carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* using lateral flow assay

We observed a 100% ($n=17/17$) detection rate of bla_{NDM}-encoded carbapenemase production among CREK bacterial strains. The test demonstrated a sensitivity and specificity of 100% (CI 80.49 – 100%). The negative predictive value (NPV) and the positive predictive value (PPV) were 100% (CI 80.49 – 100%). Moreover, the outcomes of lateral flow assays revealed that 52.9% ($n=9/17$) of *E. coli* strains exhibited co-production of bla_{OXA}, while 47% ($n=8/17$) of *K. pneumoniae* strains were identified as coproducers of bla_{OXA}($p<0.05$). Furthermore, we found a higher 82.35% ($n=14/17$) bla_{OXA} incidence among bla_{NDM}-negative *K. pneumoniae* in comparison to *E. coli* isolates 41% ($n=7/17$) ($p<0.05$) (Table 3).

Comparative analysis of phenotypic and genotypic methods for identifying carbapenemase genes in carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae*

WGS analysis revealed a comparable variant of bla_{NDM}, specifically bla_{NDM-5}, encoding carbapenemase within CREK. ($p<0.05$). In contrast, the predominant bla_{OXA} variants among *E. coli* were bla_{OXA-1} and $bla_{OXA-181}$, while *K. pneumoniae* bacterial strains predominantly carry $bla_{OXA-232}$($p<0.05$). Furthermore, the results for the subset of strains (positive for bla_{NDM}, co-producers of bla_{NDM} and bla_{OXA}, those negative for both bla_{NDM} and bla_{OXA}, and those with bla_{OXA} without bla_{NDM}) were compared across lateral flow, PCR and NGS methods. For the bla_{NDM}-positive subset, *Escherichia coli* demonstrated a sensitivity and specificity of 100% (95% CI: 80.49 – 100%), with a Kappa coefficient of 0.91 (95% CI: 0.661–1.0, $p<0.01$). The PPV and NPV were also 100% (95% CI: 80.49 – 100%). Conversely, for Klebsiella pneumoniae strains, sensitivity and specificity were 40% (95% CI: 5.27 – 85.34%), with both

PPV and NPV at 40% (95% CI: 15.50 – 70.78%). The Kappa coefficient for *K. pneumoniae* was 0.20 (95% CI: 0.104–0.298, $p < 0.01$). Conversely, for strains co-producing bla_{NDM} and bla_{OXA}, as well as those negative for both bla_{NDM} and bla_{OXA}, and those with bla_{OXA} without bla_{NDM}, we found a sensitivity and specificity of 100% (CI 80.49 – 100%), with PPV and NPV both at 100% (CI 80.49 – 100%) (Table 4). Moreover, the analysis through WGS indicated the absence of bla_{KPC}, bla_{VIM}, and bla_{IMP} within CREK isolates.

Resistome analysis and plasmid profiling of $bla_{NDM}$and bla_{OXA}carrying *Escherichia coli* and *Klebsiella pneumoniae* bacterial isolates

Across all the WGS-tested isolates, the CARD analysis revealed 370 gene variants conferring resistance to 16 distinct classes of antibiotics. The beta-lactam drug class had the highest diversity of resistant genes ($n = 67$), followed by fluoroquinolones ($n = 64$). The most prevalent beta-lactam drug resistance gene variants were bla_{ampH}, $bla_{CTX-M-15}$, bla_{OXA-1}, and bla_{NDM-5} (Fig. 3). Furthermore, IncFIB was the most prevalent plasmid among CREK (Fig. 4).

Discussion

The study was designed to investigate the accuracy and efficiency of the lateral flow kits for detecting carbapenem resistance. Among the XDR isolates included in the study, the highest frequencies of CR were observed for *K. pneumoniae*, which agrees with the literature where XDR and pan-drug resistant (PDR) *K. pneumoniae* cases have been reported around the globe [49–51]. Notably, the lowest resistance rates were observed for colistin and tigecycline, reaffirming their role as last-line treatment options, similar to the reports published worldwide, which suggest them to be the only therapeutic choice to treat infections caused by XDRs [52, 53].

The results of the present study indicate a high incidence of CREK associated with urinary tract infections (UTIs), which is comparable with other studies [54, 55]. These findings imply that CREK could potentially play a role in causing UTIs among hospitalized patients. However the findings contrasts with a surveillance data from the region, where pus specimens were reported as the most common source of CREK isolates [56].

New Delhi Metallo-beta-lactamase (NDM) is the most prevalent carbapenemase in Pakistan and the broader South Asian subcontinent, reflecting a significant regional challenge in combating antimicrobial resistance [57–59]. In alignment with these trends, our study identified NDM as the dominant carbapenemase among the isolates, followed by OXA, VIM, and IMP, with KPC being the least frequently observed resistance mechanism [8]. These findings highlight the utility of targeted diagnostic approaches in this context. Specifically, lateral flow assays, such as RESIST-4 OKNV or RESIST-3 OKN, could serve as cost-effective alternatives for detecting carbapenemase activity in clinical settings. The implementation of these streamlined assays has the potential to significantly improve diagnostic accessibility and cost-effectiveness, particularly in resource-limited settings, while maintaining a high standard of diagnostic accuracy.

However, when evaluating bla_{NDM} detection using statistical tests, the sensitivity and specificity of the studied lateral flow kits were 100% for *E. coli* compared to PCR, which was considered the gold standard for gene detection. These results coincide with other studies using the CORIS Bio Concept, Gembloux, Belgium kits [60–63]. While *K. pneumoniae* showed a

Table 4 Comparative analysis of phenotypic vs. genotypic methods in carbapenemase gene identification - overall performance Metrics

Carbapenem-resistant bacterial strains	PCR and Lateral flow categories	Subcategories	NGS		PPV %	NPV%	Kappa (CI 95%)
			Detection	Variants			
E. coli	Positive	bla_{NDM} ($n = 5$)	5 (100%)	bla_{NDM-5}	100	100	0.91[0.66 – 1]
		co-producers of bla_{NDM} and bla_{OXA} ($n = 5$)	5 (100%)	bla_{OXA-1}, $bla_{OXA-181}$	100	100	0.91[0.66 – 1]
	Negative	bla_{NDM} and bla_{OXA} ($n = 2$)	Not detected				
		bla_{OXA} without bla_{NDM} ($n = 2$)	2 (100%)	$bla_{OXA-181}$	100	100	0.34[0.15 – 0.55]
K. pneumoniae	Positive	bla_{NDM} ($n = 5$)	2 (40%)	bla_{NDM-5}	40	40	0.20[0.10 – 0.2]*
		co-producers of bla_{NDM} and bla_{OXA} ($n = 5$)	5 (100%)	$bla_{OXA-232}$	100	100	0.91[0.66 – 1]
	Negative	bla_{NDM} and bla_{OXA} ($n = 2$)	Not detected				
		bla_{OXA} without bla_{NDM} ($n = 2$)	2 (100%)	$bla_{OXA-232}$	100	100	0.34[0.15 – 0.55]

The comparative effectiveness of phenotypic (lateral flow) and genotypic (PCR) methods was assessed through whole genome sequencing to detect the presence of the New Delhi Metallo-beta-lactamases (bla_{NDM}) gene in carbapenem-resistant Escherichia coli and Klebsiella pneumoniae (CREK). Numbers expressed in percentage (%). PPV: Positive Predictive Value; NPV: Negative Predictive Value

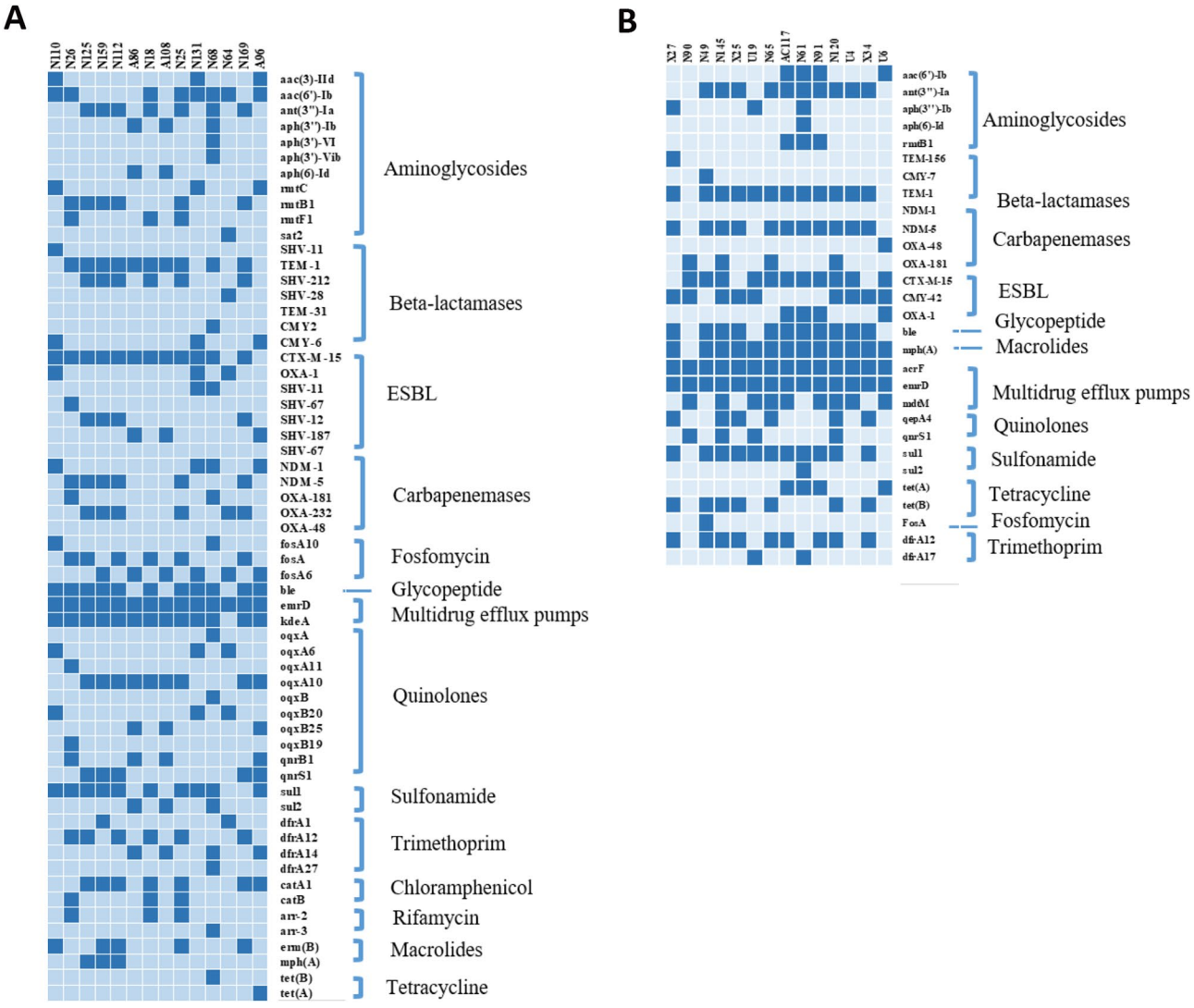

Fig. 3 Antibiotic resistance gene profiling. The antibiotic resistance genes (ARGs) in carbapenem-resistant *K. pneumoniae*(**A**) and *E. coli*(**B**) (CREK) isolates carrying bla_{NDM} and bla_{OXA} were identified by aligning their whole-genome sequences with the Comprehensive Antibiotic Resistance Database (CARD). Dark blue blocks represent ARGs exhibiting 70% identity. Further, ARGs were categorized based on their functions, and the respective categories are presented alongside the gene name

lower sensitivity of 40%, indicating that the kits may have failed to identify NDM-producing strains despite PCR confirmation of the presence of bla_{NDM} gene. The discrepancies in the results could have been because of the late expression or the drug-induced expression of the bla_{NDM} gene [61, 64]. However, these findings need further investigation and present the study's limitations.

Additionally, the resistome analysis indicated that beta-lactam genes were the most prevalent among all CREK, which coincides with the results of the antibiotic susceptibility testing presenting high resistance against beta-lactams. Interestingly, colistin resistance was observed in CREK isolates even without a mobile colistin resistance (mcr) gene, which is typically responsible for encoding colistin resistance and was not identified during CARD analysis [65]. These results suggest the possible acquisition of acquired resistance to colistin. As reported by early studies, lipopolysaccharide (LPS) modification may develop colistin resistance [66, 67]. The chromosomal genes *phoPQ*, *pmrAB*, and *mgrB* were the reported cause of colistin resistance until the first report of the *mcr* genes [68, 69]. None of these genes were found during the CARD analysis, these findings indicate that isolates with phenotypic colistin resistance must be subjected to further testing to identify the underlying mechanisms of colistin resistance.

The study reveals that the lateral flow assays tested for detecting bla_{NDM} genes, which are responsible for

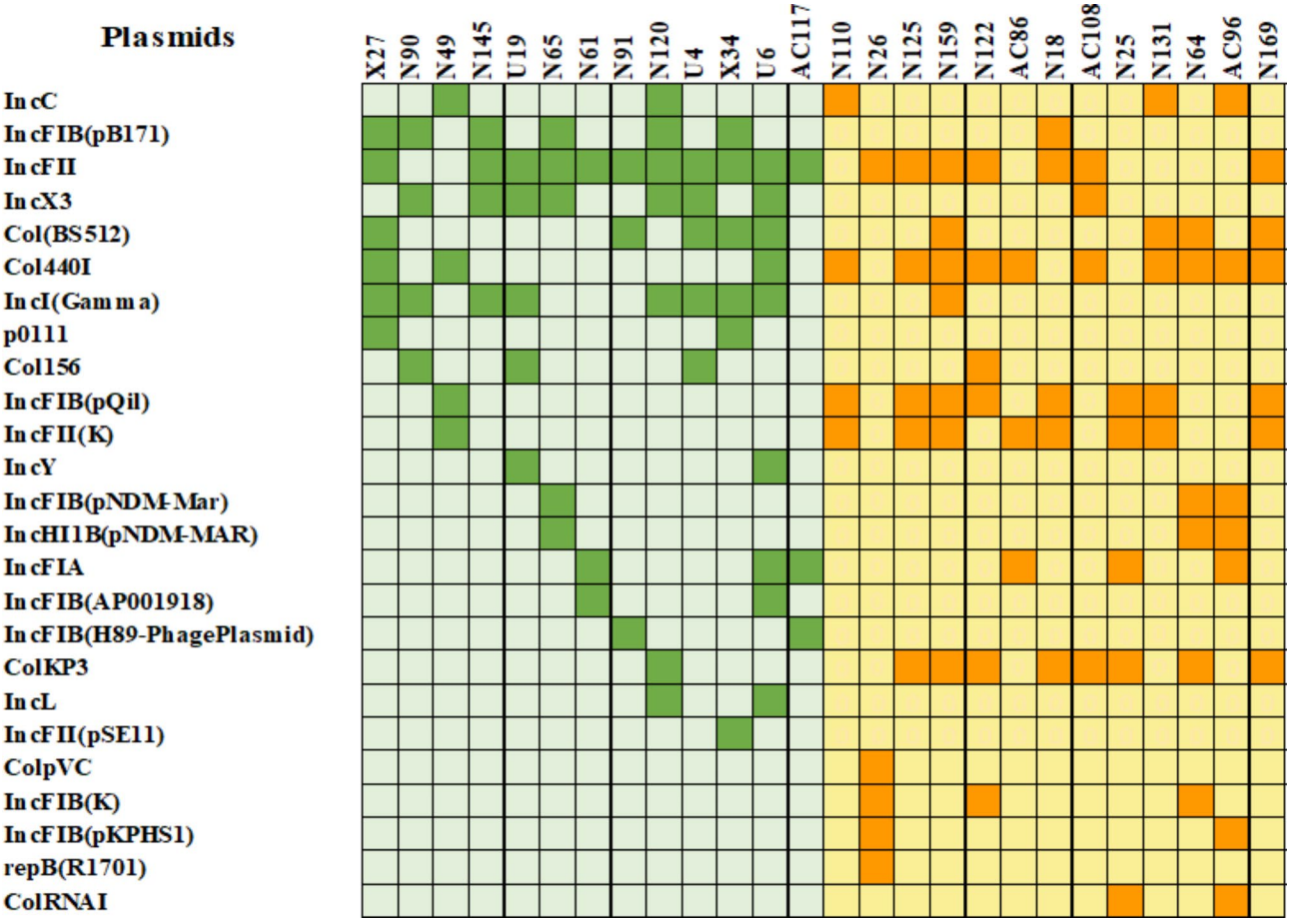

Fig. 4 Plasmid maps profiling of bla_{NDM}, Plasmids were generated using nanopore sequencing data. The dark green colored blocks present the plasmids for *E. coli* and dark orange coloured blocks present plasmids for *K. pneumoniae.*

carbapenemase production, show promise as a reliable and rapid diagnostic tool for E. coli bacterial strains. This advancement has the potential to significantly enhance clinical diagnostics and patient outcomes by enabling timely and accurate detection of carbapenem-resistant bla_{NDM}-encoding *E. coli* infections.

Supplementary Information

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Supplementary Material 1

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Author contributions
Noor. Ain: data curation, formal analysis, investigation, methodology, Resources, Software, validation, visualization, and writing of the original draft. S. Riaz and T. McHugh: Study design Review, editing, supervision, validation. L. Elton: WGS methodology, editing. SZ: MALDI and lateral flow analysis.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval
The local ethics committee approved the study (CitiLab and Research Centre Ref # 30th – 15 CLRC/ 30th).

Consent to participate
This study does not involve any patients.

Competing interests
The authors declare no competing interests.

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