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RESEARCH ARTICLE

Molecular characterization of carbapenem resistant *E. coli* of fish origin reveals the dissemination of NDM-5 in freshwater aquaculture environment by the high risk clone ST167 and ST361

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Abstract

Aquatic environment can act as reservoir and disseminator of antimicrobial resistance and resistant pathogens. Novel high-risk carbapenem resistant *E. coli* (CREC) are continuously emerging worldwide; however, the occurrence of CREC in freshwater aquaculture environment is largely unexplored. To fill this gap, large scale sampling of freshwater pond sites and retail fish markets was done between Oct 2020 and Oct 2021 to investigate the CREC contamination in fish. The frequency of CREC contamination in the freshwater fish was 6.99% (95% CI: 3.78–10.20%). All the isolates were MDR and harbored carbapenemase encoding gene, $bla_{\text{NDM-5}}$ along with other antimicrobial resistance genes (ARGs), bla_{TEM} (64.7%), $bla_{\text{CTX-M-15}}$ (35.3%), $bla_{\text{OXA-1}}$ (5.9%), *tet(A)* (100%), *sul1* (94.1%), *qnrS* (82.3%), *cat1* (35.3%), and *cat2* (23.5%). The isolates belonged to phylogroup C and showed low virulence gene profile. ERIC-PCR grouped the isolates into five clusters (I-V). The isolates of clusters I, II, and III were identified as ST167 (76.4%) and of cluster IV as ST361 (17.6%). This is the first report documenting the contamination of NDM-5 producing *E. coli* ST167 and ST361 of clinical/livestock lineage in freshwater fish from India. The $bla_{\text{NDM-5}}$ was significantly associated with ARGs, *tet(A)*, and *sul1*; and plasmid replicons, IncF, IncI1, and IncP, signifying the presence of $bla_{\text{NDM-5}}$ and associated ARGs on these transferable plasmids. These findings were validated by the successful conjugal transfer of $bla_{\text{NDM-5}}$ and associated ARGs into non-CREC strain (J53). Our study highlights the ability of CREC to disseminate antimicrobial resistance which has health implications and environmental concerns.

Keywords Carbapenem resistant *E. coli* · Freshwater aquaculture environment · Antimicrobial resistance genes (ARGs) · $bla_{\text{NDM-5}}$ · Sequence types (STs) · Incompatible plasmid replicons

Introduction

Carbapenems are considered the last effective antimicrobial drugs to treat infections caused by extended spectrum β-lactamase (ESBL) producing Gram-negative bacteria (van Duin and Doi 2017). *E. coli* is a commensal as well as pathobiont causing intestinal and extra-intestinal infections in humans and livestock (Denamur et al. 2021). Carbapenem-resistant *E. coli* (CREC) is recognized as one of the major public health concerns that is primarily mediated by the production of carbapenemases (Gupta et al. 2011). The common antimicrobial resistance genes (ARGs) encoding for the production of carbapenemase are bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and $bla_{\text{oxa-48}}$ (Queenan & Bush 2007). New Delhi metallo-β-lactamase is one of the most widely reported carbapenemase which is encoded by bla_{NDM} gene (Khan et al. 2017). Till 2020, 28 variants of bla_{NDM} gene have been identified among different bacterial species globally (Farhat and Khan 2020). Among them, $bla_{\text{NDM-5}}$ shows extensive hydrolytic activity toward carbapenems and β-lactams along with higher transferability through plasmid (Hornsey et al. 2011). Several other genes encoding resistance to cephalosporins (bla_{TEM}, $bla_{\text{CTX-M-15}}$),

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tetracyclines (*tet(A)* and *tet(B)*), quinolones (*qnrS, qnrB*), aminoglycosides (*aadA1*, *aac(6')-Ib-cr*), and sulphonamides (*sul1* and *sul2*) have also been detected in bla_{NDM-5} harboring Enterobacteriaceae (Liang et al. 2018; Chowdhury et al. 2022). The higher use of carbapenems in clinical practice has led to a rise in the prevalence of CREC in clinical settings (Gupta et al. 2011), livestock ((Köck et al. 2018), and the environment (Cherak et al. 2021). The majority of the CREC isolated from clinical cases harbor the bla_{NDM-5} on transferable plasmids of diverse incompatible (Inc) replicon types, such as IncF, IncFII, and IncX3 (Kopotsa et al. 2019). There exists a wide genomic diversity in CREC, and the most commonly reported sequence types (STs) among NDM producing *E. coli* are ST101, ST167, ST131, ST405, ST410, and ST648 (Dadashi et al. 2019). The various STs reported in NDM producing CREC isolated from clinical specimens in India are ST167, ST101, ST131, ST648, ST405, and ST410 (Devanga Ragupathi et al. 2020; Paul et al. 2020). Additionally, CREC has also been detected in food-producing terrestrial animals and companion animals from India (Köck et al. 2018; Bandyopadhyay et al. 2021)

India is the second-largest producer of farmed fish after China. Inland finfish species in India together account for 6.3 million tonnes (89.2%), out of total production of 7.1 million tonnes (FAO 2020). This signifies the importance of the freshwater aquatic environment as a major contributor of farmed fish. Contamination with antimicrobials in the aquatic environment will affect both the quality and quantity of the farmed fish (Cabello et al. 2016). The aquatic environment is vulnerable to contamination with antimicrobials mainly by anthropogenic activities. Kumar et al. (2022a) observed traces of feces on the pond dykes, incoming household drainage, and animal ingression in the freshwater aquaculture environment of Uttar Pradesh, India. The contamination with antimicrobials in the aquatic environment will not only affect the bacterial diversity in the ecosystem but could also act as a driver for the emergence of antimicrobial resistance (AMR). Thus, the aquatic environment serves as a common pool of AMR and aids in its dissemination through the food chain (Cabello et al. 2016). Besides this, the food fish produced in such an environment can also be a public health concern. Many countries have reported the presence of CREC in aquatic environment and seafood (Cherak et al. 2021; Singh et al. 2016; Das et al. 2019). However, relatively few studies have been conducted to determine the occurrence of CREC in fish of the freshwater environment (Hamza et al. 2020; Nakayama et al. 2022). In India, the data on the presence of CREC in fish and its characterization is also limited to a few reports only. Two isolates of CREC were documented in seafood sold in the retail fish market, which was characterized as ST131 belonging to phylogroup B2 (Singh et al. 2016; Das et al. 2019). In another study, eight CRECs were isolated from two fish farms which were adjacent to water bodies receiving hospital effluents in Kerala, India (Kalasseril et al. 2020). All these CRECs were negative for carbapenemase encoding genes, bla_{NDM-1}, bla_{VIM}, and bla_{IMP}. In this context, assessing the contamination of CREC in fish of freshwater aquaculture environment is necessary to prevent the dissemination of carbapenem resistance. Accordingly, this study is aimed at (i) determining the frequency of CREC contamination in fish of freshwater environment from an aquaculture area in Northern India, (ii) identifying the molecular mechanism of AMR, (iii) characterizing the CREC to determine the STs, phylogroups, virulence gene profile, and incompatible (Inc) plasmid replicons, (iv) examining whether the carbapenem resistance in *E.coli* is associated with other AMR phenotypes, ARGs, and/or Inc plasmid replicons, and (v) assessing the potential of CREC to disseminate AMR by conjugal transfer.

Materials and methods

Sample collection

The study was carried out in Uttar Pradesh, which is the largest aquaculture producing state in Northern India. The sampling was conducted from 175 pond sites of freshwater aquaculture environment and 6 retail fish markets in seven aquaculture dominating districts (Supplementary figure). A total of 243 gut swabs from freshwater fish (at least ~ 200 g) were collected from October 2020 to October 2021. The fish species included pangas (*Pangasianodon hypophthalmus*, $n = 98$), rohu (*Labeo rohita*, $n = 61$), mrigal (*Cirrhinus mrigala*, $n = 35$), silver carp (*Hypophthalmichthys molitrix*, $n = 19$), grass carp (*Ctenopharyngodon idella*, $n = 12$), common carp (*Cyprinus carpio*, $n = 12$), and catla (*Catla catla*, $n = 6$). Aseptically, fish were dissected at the site and the hind gut of 15–20 cm in length from each fish was cut open and swabbed by Amies transport swab (HiMedia, India). The swabs were brought to the laboratory on ice for bacterial isolation.

Isolation of CREC

The swabs were inoculated in tryptic soya broth (TSB) containing meropenem at a final concentration of 2 µg/ml and incubated for 18 h at 37 °C with shaking at 200 rpm. Enriched bacterial culture was diluted and spread plated onto a violet red bile glucose agar (VRBGA) plate supplemented with meropenem (2 µg/ml). Three pink color colonies showing oxidase negative reaction were picked from VRBGA and streaked on an EMB agar plate followed by overnight incubation at 37 °C. Bacterial colonies that produced metallic sheen on EMB agar were purified on nutrient agar (NA) and

subjected to standard biochemical tests for the preliminary identification of *E. coli* (Bandyopadhyay et al. 2021). The molecular confirmation of *E. coli* was accomplished by the screening of the *uidA* gene (Gómez-Duarte et al. 2010). *E. coli* ATCC 25922 was used as a control in the study. The isolates which were non-susceptible to meropenem at a concentration of 2 µg/ml (CLSI 2021) were considered CREC. The isolates were preserved in 20% glycerol and stored at -80 °C.

Phenotypic testing for AMR

Antibiotic susceptibility testing (AST)

Antimicrobial susceptibility testing of CREC was performed by Kirby–Bauer disk diffusion method with some modifications (Hudzicki 2009). The tested antimicrobial agents were imipenem (10 µg), meropenem (10 µg), cefoxitin (30 µg), ceftriaxone (30 µg), ceftazidime (5 µg), cefotaxime (10 µg), cefepime (30 µg), gentamicin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), and co-trimoxazole (1.25/23.75 µg). The results were interpreted as per the CLSI breakpoints (CLSI 2021). *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as quality control strains for AST. The isolates that were resistant to three or more classes of antimicrobials were considered as multidrug resistant (MDR).

ESBL and AmpC β-lactamase production (ACBL)

The CREC that was phenotypically resistant to any of the third generation cephalosporins (ceftriaxone, ceftazidime, and cefotaxime) and cefoxitin was tested for the production of ESBL and ACBL, respectively (Peter-Getzlaff et al. 2011).

Carbapenemase production

Carbapenemase production was tested by the modified carbapenem inactivation method (mCIM). Carbapenemase producers were further tested by the EDTA-modified carbapenem inactivation method (eCIM), to differentiate between serine and metallo-β-lactamase producers (Sfeir et al. 2019).

Minimum inhibitory concentration determination (MIC)

The MIC of meropenem for CREC was determined by the agar dilution method (CLSI 2015) and further MIC_{50} and MIC_{90} were calculated as per the method given by Schwarz et al. (2010).

Molecular detection of ARGs and sequence analysis

The resistant phenotypes were screened for their corresponding ARGs by PCR. Briefly, rapid DNA extraction was performed from bacterial cell suspension as described elsewhere (Dallenne et al. 2010). Total DNA (2 µl) was used as a template in the PCR reaction for the detection of 24 ARGs which included bla_{NDM} (Manchanda et al. 2011), bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{GES}, bla_{OXA-48}, bla_{SHV}, bla_{TEM}, bla_{OXA-1}, $bla_{CTX-M\ group\ 1}$, $bla_{CTX-M\ group\ 2}$, and $bla_{CTX-M\ group\ 9}$ (Dallenne et al. 2010), *tet(A)*, *tet(B)*, *tet(G)*, *tet(G1)* (Ng et al. 2001), *qnrA*, *qnrB*, *qnrS*, *qnrC* (Kim et al. 2009), *sul1* (Zhao et al. 2001), *dhfr1* (Ture et al. 2018), *cat1* and *cat2* (Yoo et al. 2003). The final volume of the PCR mixture was 25 µl containing 1X PCR buffer, 200 µM dNTPs, 1.6 mM $MgCl_2$, 1.25 U Taq DNA polymerase, and 0.2–0.4 µM of primers. The PCR amplicons of bla_{NDM} and $bla_{CTX-M\ group}$ genes were sequenced by the sanger dideoxy method to determine the allelic variant of the enzymes. The obtained nucleotide sequences were compared with the gene sequences of the GenBank database using the blastn algorithm to determine their relatedness to other nucleotide sequences.

Molecular characterization of CREC

Clonal relatedness by enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

The ERIC-PCR was done to determine the clonal relatedness of CREC by using ERIC1R-ATGTAAGCTCCTGGGGATTCAC and ERIC2-AAGTAAGTGACTGGGGTGAGCG primers (Versalovic et al. 1991). Briefly, the PCR reaction of 25 µl containing 1xPCR buffer, 200 µM dNTPs, 4 mM $MgCl_2$, 2.5 U Taq polymerase, and 0.2 µM of each primer was performed at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 5 min, and a final extension at 72 °C for 16 min. The amplified products were visualized in 1.2% agarose gel in 0.5xTAE buffer with 1 kb ladder after running at 60 V for 4 h. The gel was photographed and the dendrogram was constructed in GelClust using the UPGMA clustering method and dice distance coefficient (Khakabimamaghani et al. 2013).

Phylogrouping

Clermont quadruplex PCR was used to categorize CREC isolates into phylogroups A, B1, B2, C, D, E, F and cryptic clade I using the primers *chuA*, *yjaA*, *TspE4.C2*, *AceK*, *ArpA1* and *trpAgpC* (Clermont et al. 2013).

Multi-locus sequence typing (MLST)

The MLST was performed by amplifying 7 housekeeping genes of *E. coli* (*adk*, *fumC*, *icd*, *purA*, *gyrB*, *mdh* and *recA*) according to the EnteroBase (https://enterobase.warwick.ac.uk/). To identify the sequence type of CREC, the nucleotide sequence of each gene was searched in the pubmlst database to generate an allelic number and the sequence type for each isolate.

Detection of virulence genes

The CREC were screened by PCR for 6 entero-pathogenicity genes (Abbasi et al. 2020) and 12 extra-intestinal virulence genes (Ewers et al. 2007). The entero-pathogenicity genes were attaching and effacing gene (*eae*) for enteropathogenic *E. coli* (EPEC), Shiga toxin gene (*stx*) for enterohemorrhagic *E. coli* (EHEC), heat labile and heat stable enterotoxin gene (*elt* and *st*) for enterotoxigenic *E. coli* (ETEC), aggregation associated plasmid (*pCVD432*) for enteroaggregative *E. coli* (EAEC), and Invasion-associated locus (*ial*) for enteroinvasive *E. coli* (EIEC). The extra-intestinal virulence genes were type-1 fimbrial adhesion gene (*fimC*), invasion gene (*ibeA*), siderophore receptor gene (*iroN*), temperature sensitive hemagglutinin (*tsh*), cytotoxic necrotizing factor gene (*Cnf1/2*), hemolysins A gene (*hlyA*), serine protease autotransporters (*sat*), iron-responsive element (*ireA*), iron repressible protein (*irp2*), meningitis-associated and temperature regulated fimbriae (*mat*), genetic island associated with newborn meningitis (*gimB*), and structural genes of colicin V operon (*cvi/cva*).

Plasmid-based replicon typing (PBRT)

A total of 18 Inc plasmid replicons were screened to identify the types of plasmids present in the CREC. The plasmid Inc replicons FIA, FIB, FIC, HI1, HI2, I1, L/M, N, P, W, T, A/C, K/B, B/O, X, Y, F and FIIA were screened by five multiplex and three simplex PCR as per Carattoli et al. (2005).

Conjugal transfer of AMR

The potential of CREC to transfer the carbapenem and/or associated ARGs into the *E. coli* J53 (azide resistant-recipient strain) was evaluated using a broth mating experiment following the procedure given by Das et al. (2019). The trans-conjugants were screened for ARGs by PCR along with phenotypic testing of AMR (AST and MIC) as per the method described above.

Statistical analysis

The difference in the proportion of CREC contamination between fish of freshwater aquaculture environment and retail fish markets was analyzed by two sample *Z*-test. The MIC of meropenem for CREC was compared by the Mann–Whitney *U* test between these two sources. These statistical analyses were performed by SPSS version 16.0 at a significance level of 5% ($p < 0.05$). Phi correlation analysis was conducted to (i) determine the association of ARGs with corresponding resistant phenotypes and (ii) examine whether the carbapenem resistance in *E.coli* is associated with any other AMR phenotypes, ARGs, and/or Inc plasmid replicons. For this, the obtained data were converted into binary variables and analyzed according to Osman et al. (2018) and its correlation matrix was plotted with R software (R-4.2.1) using corrplot package at a significance level of 5% ($p < 0.05$).

Results

Frequency of CREC

Out of the 243 samples screened, seventeen were positive for CREC indicating an overall frequency of 6.99% (95% CI: 3.78–10.20%). Significantly higher ($p < 0.05$) contamination of CREC was noted in retail fish markets than in freshwater aquaculture environment. The positivity rate of CREC for samples from freshwater aquaculture environment was 4% (95% CI: 1.08–6.91%) as compared to 14.71% (95% CI: 6.22–23.19%) for samples from retail fish markets. Overall, 41 isolates were identified as CREC based on colony morphology and biochemical characteristics (Gram-negative, motile, oxidase-negative, catalase-positive, and producing metallic sheen growth on EMB agar). All the isolates were re-confirmed as *E. coli* by PCR amplification of *uidA* gene. One isolate from each sample positive for CREC (a total of 17 non-repetitive) was selected for further characterization. The frequency of CREC is given in Table 1.

AMR profile

The CREC were resistant to meropenem, cephalosporins (cefoxitin, ceftazidime, cefotaxime, ceftriaxone, and cefepime), tetracycline, and enrofloxacin. Resistance to imipenem and co-trimoxazole was observed in 94.1% of the isolates. Non-susceptibility to gentamicin and chloramphenicol was observed in 47% and 35.3% of the isolates, respectively. All the isolates were MDR as they showed resistance to three or more different classes of antimicrobials. The AMR profile of CREC is depicted in Fig. 1.

Table 1 Frequency of carbapenem resistant *E. coli* (CREC) contamination in fish of freshwater aquaculture environment and retail fish markets

Source of the sample	Number of samples screened	Number of samples positive for CREC (%)	95% confidence interval (CI)
Freshwater aquaculture environment	175	7 (4.0%)	1.08–6.91%
Retail fish markets	68	10* (14.7%)	6.22–23.19%
Total	243	17 (6.99%)	3.78–10.20%

*Indicates significant difference ($p < 0.05$)

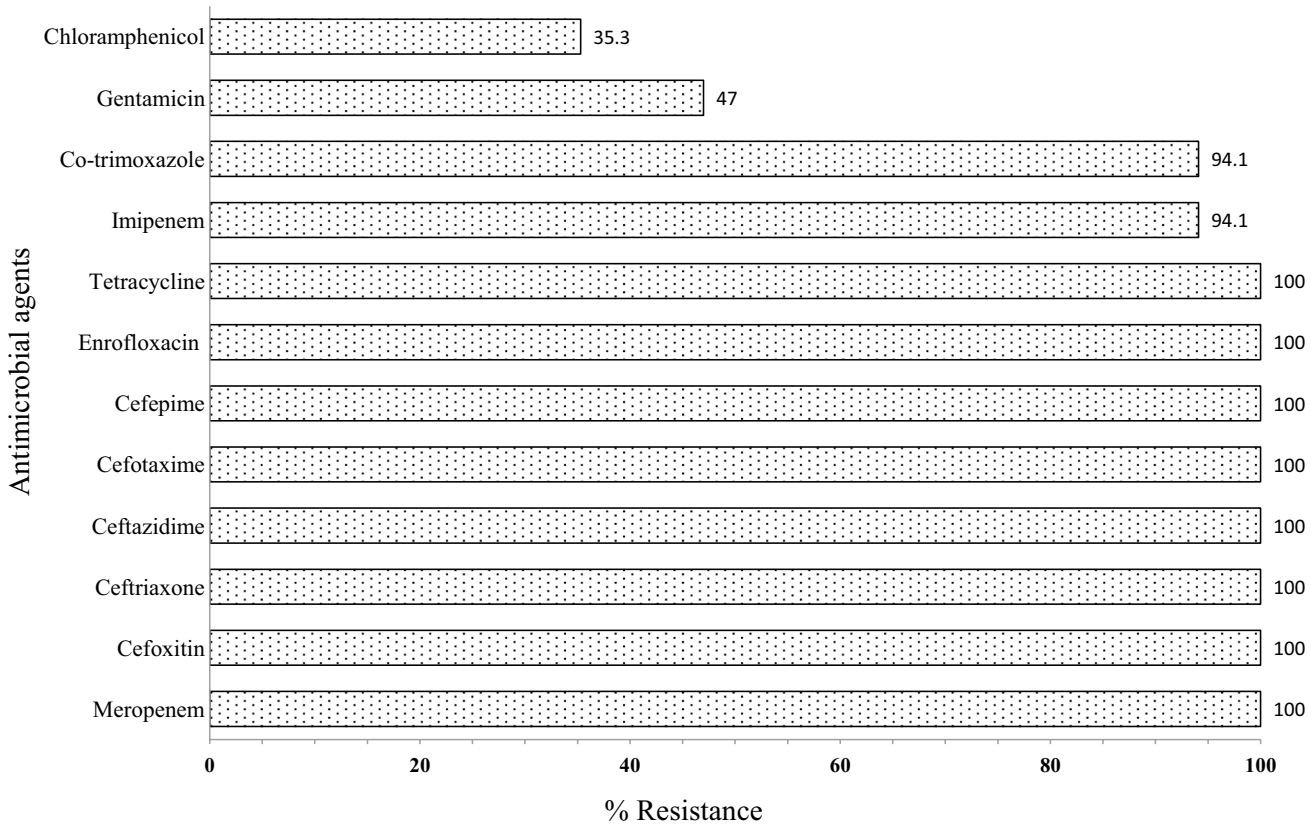

Fig. 1 Antimicrobial resistance profile of carbapenem resistant *E. coli* (CREC)

Production of ESBL, ACBL, and carbapenemase

None of the CREC was positive for ESBL and ACBL production by double disk synergy test. All the isolates were positive for the production of carbapenemase and metallo-β-lactamase (class B carbapenemase) by mCIM and eCIM.

MIC

The MIC of meropenem for CREC ranged from 8 to 128 µg/ml. Overall, 47% of CREC had MIC of 64 µg/ml, while 29.4% of the isolates showed MIC of 128 µg/ml. The MIC_{50} of the isolates from freshwater aquaculture environment was lower (32 µg/ml) than the isolates from retail fish markets (64 µg/ml), whereas the MIC_{90} was 128 µg/ml, irrespective of the source (Table 2). The MIC of cefotaxime, ciprofloxacin, and tetracycline was ≥ 64 µg/ml, ≥ 4 µg/ml, and ≥ 64 µg/ml, respectively. Similarly, the MIC of ampicillin was ≥ 64 µg/ml for 94.1% of the isolates, whereas the MIC of gentamicin was ≥ 64 µg/ml for 47% of the isolates. Further, the MIC of chloramphenicol ranged between 8 µg/ml and 32 µg/ml (Table 3).

ARGs

Among the six carbapenemase encoding genes screened (bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{GES}, and bla_{OXA-48}), only

Table 2 MIC_{50} and MIC_{90} of meropenem for carbapenem resistant *E. coli* (CREC) from fish of freshwater aquaculture environment and retail fish markets

Source (n = number of isolates)	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Freshwater aquaculture environment ($n = 7$)	8–128	32	128
Retail market fish ($n = 10$)	64–128*	64	128

*Indicates significantly different ($p < 0.05$)

Table 3 The minimum inhibitory concentration (MIC) of antimicrobials for carbapenem resistant *E. coli* (CREC) and corresponding transconjugants (TC)

Isolates	MIC (μg/ml)							
	MEM	AMP	CTX	CIP	GEN	TET	CHL	Sodium azide
Donor-CREC-1	128 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	8 (S)	≤32
TC-CREC-1	32 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≥64 (R)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-2	64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	8 (S)	≤32
TC-CREC-2	8 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-3	128 (R)	32 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	8 (S)	64
TC-CREC-3	128 (R)	32 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	8 (S)	≥1024
Donor-CREC-4	128 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	8 (S)	≤32
TC-CREC-4	32 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-5	64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	16 (I)	64
TC-CREC-5	8 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≥64 (R)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-6	128 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	16 (I)	≤32
TC-CREC-6	128 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≥64 (R)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-7	64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	8 (S)	64
TC-CREC-7	8 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-8	128 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	16 (I)	≤32
TC-CREC-8	16 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≥64 (R)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-9	8 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	32 (R)	≤32
TC-CREC-9	4 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≥64 (R)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-10	16 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	8 (S)	≤32
TC-CREC-10	4 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-11	64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	8 (S)	≤32
TC-CREC-11	8 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-12	32 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	32 (R)	≤32
TC-CREC-12	8 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≥64 (R)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-13	64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥64 (R)	≥64 (R)	8 (S)	≤32
TC-CREC-13	8 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≥64 (R)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-14	8 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	16 (I)	64
TC-CREC-14	4 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-15	64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	8 (S)	64
TC-CREC-15	4 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-16	64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	8 (S)	64
TC-CREC-16	8 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Donor-CREC-17	64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≤1 (S)	≥64 (R)	8 (S)	≤32
TC-CREC-17	8 (R)	≥64 (R)	≥64 (R)	≤0.125 (S)	≤1 (S)	≥64 (R)	≤4 (S)	≥1024
Recipient *E. coli* J53	≤0.5 (S)	≤2 (S)	≤0.5 (S)	≤0.125 (S)	≤1 (S)	≤2 (S)	≤4 (S)	≥1024

MEM meropenem, *AMP* ampicillin, *CTX* cefotaxime, *CIP* ciprofloxacin, *GEN* gentamicin, *TET* tetracycline, *CHL* chloramphenicol

bla_{NDM} was detected in all the CREC. In addition, bla_{TEM}, $bla_{CTX\text{-}M\ group\ 1}$ and $bla_{OXA\text{-}1}$ were detected in 64.7%, 35.3%, and 5.9% of the isolates, respectively. The other ARGs detected were *tet(A)* (100%), *sul1* (94.1%), *qnrS* (82.3%), *cat1* (35.3%) and *cat2* (23.5%), respectively. The distribution of ARGs in CREC and their association with the corresponding resistance phenotypes is given in Table 4.

The PCR amplicons of bla_{NDM} and $bla_{CTX\text{-}M\ group\ 1}$ from representative isolates were sequenced to determine their variants. Nucleotide blast search analysis revealed that the CREC harbored $bla_{NDM\text{-}5}$ and $bla_{CTX\text{-}M\text{-}15}$ gene variants. The $bla_{NDM\text{-}5}$ gene sequences of CREC and its NDM-5 protein revealed > 99% similarity with NDM-5 sequences available in the GenBank database of NCBI. The obtained nucleotide sequences were submitted to NCBI with accession numbers OP081826, OP081828, OP081830, OP081831, and OP081835.

Molecular characterization

Diversity analysis of CREC by ERIC-PCR showed amplicons ranging between 200 and 2500 bp, and the isolates dispersed into 5 clusters on the basis of different genetic patterns. Among the 8 phylogroups screened (A, B1, B2, C, D, E, F, and cryptic clade I), all the CREC was identified as phylogroup C. MLST analysis of CREC indicated that the isolates of cluster I, II, and III ($n = 13$ and 76.4%) belonged to ST167. The isolates of cluster IV belonged to ST361 (17.6%), while the MLST pattern of CREC-17 did not match with any of the reported STs. The MLST gene sequences were submitted to NCBI with accession numbers OP131734-OP131736, OP131738-OP131742, OP131744, OP131745, OP131747-OP131751, OP131753-OP131759, OP131761-OP131766, and OP131768-OP131774.

Among the 18 virulence genes screened, *mat* was detected in all the isolates (100%), whereas *fim*C was detected in 17.6% of the isolates that belonged to ST361. The rest of the sixteen virulence genes were not detected. Nine Inc plasmid replicons were identified in the CREC. The detected plasmid replicon types were IncF (100%), IncI1, and IncP (94.1%, each) followed by IncK/B (47%), IncY (17.6%), IncB/O (11.8), IncFIA (11.8%), IncFIB (5.9%), and IncN (5.9%). A total of 12 different replicon combinations were observed and each isolate harbored at least two replicons. The IncF + IncI1 + IncP replicon was the most frequent combination which was detected in 88.23% of the isolates (Fig. 2).

Conjugal transfer

Conjugation was performed to investigate the potential of the CREC to transfer bla_{NDM-5} and associated ARGs into the recipient strain. The conjugation frequency ranged between 1.5×10^{-6} and 7.2×10^{-7} transconjugants per recipient cell. The transconjugants showed increased MIC for meropenem, ampicillin, cefotaxime, tetracycline, and gentamicin relative to the recipient. However, no change was observed in the MIC for chloramphenicol. There was no increase in the MIC of ciprofloxacin for all the transconjugants, except TC-CREC-3 (Table 3). The resistant phenotypes of transconjugants were positive for their corresponding ARGs (bla_{NDM-5}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$, *tet(A)*, *qnr*S, and *sul*1).

Table 4 Distribution of antimicrobial resistance genes (ARGs) in carbapenem resistant *E. coli* (CREC) and their association with the corresponding resistance phenotypes

Antimicrobial-resistant genes profile				Association between resistant phenotypes and ARGs	
Antimicrobial classes	Number (%) of resistant phenotypes	ARGs	Number (%) of CREC harboring corresponding ARGs	Phi correlation coefficient	p (< 0.05) value
Carbapenems	17 (100)	$bla_{NDM\text{-}5}$	17 (100)	1.0	0.00*
Cephalosporins	17 (100)	bla_{TEM}	11 (64.7)	0.304	0.19
		$bla_{CTX\text{-}M\text{-}15}$	6 (35.2)	0.171	0.47
		$bla_{OXA\text{-}1}$	1 (5.9)	0.059	0.80
Tetracyclines	17 (100)	*tet(A)*	17 (100)	1.00	0.00*
Quinolones	17 (100)	*qnrS*	14 (82.3)	0.454	0.054
Sulfonamides	16 (94.1)	*sul1*	16 (100)	1.00	0.00*
Phenicols	6 (35.2)	*cat1*	2 (33.3)	0.50	0.03*
		cat1 + *cat2*	4 (66.7)	0.756	0.001*

p value (< 0.05) shows a significant association between resistance phenotypes and their corresponding ARGs

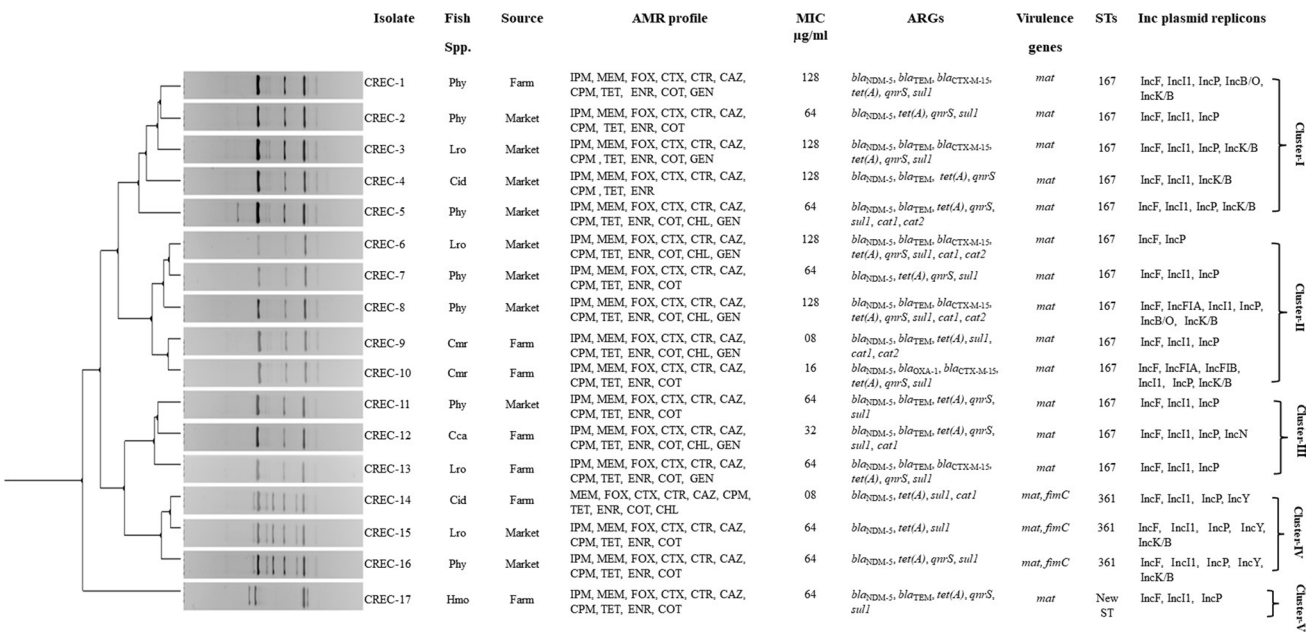

Fig. 2 Dendrogram of ERIC-PCR banding pattern representing the genetic relationship among the carbapenem resistant *E. coli* along with their phenotypic and genotypic characteristics. Fish species (Phy, *Pangasianodon hypophthalmus*; Lro, *Labeo rohita*; Cmr, *Cirrhinus mrigala*; Hmo, *Hypophthalmichthys molitrix*; Cid, *Ctenopharyngodon idella*; Cca, *Catla catla*); antimicrobial agents (IPM, imipenem; MEM, meropenem; FOX, cefoxitin; CTX, cefotaxime; CTR, ceftriaxone; CAZ, ceftazidime; CPM, cefepime; COT, co-trimoxazole; TET, tetracycline; CHL, chloramphenicol; GEN, gentamicin; ENR, enrofloxacin)

Correlation analysis

The phi correlation (r) matrix revealed that meropenem resistant phenotypes of *E. coli* showed significant positive association with AMR phenotypes, cephalosporins ($r=1.0$), tetracycline ($r=1.0$), enrofloxacin ($r=1.0$), and co-trimoxazole ($r=0.68$). These phenotypes also showed a significant positive association with $bla_{\text{NDM-5}}$ ($r=1.0$), *tet(A)* ($r=1.0$), and *sul1* ($r=0.68$). The $bla_{\text{NDM-5}}$ was significantly ($p<0.05$) associated with Inc plasmid replicons, IncF ($r=1.0$), IncI ($r=0.68$), and IncP ($r=0.68$). Cephalosporin resistance in CREC was not significantly associated with the β-lactamase encoding genes namely bla_{TEM}, $bla_{\text{CTX-M-15}}$, and $bla_{\text{OXA-1}}$. Similarly, enrofloxacin and meropenem co-resistant phenotypes showed a non-significant association with the *qnrS* gene (Fig. 3).

Discussion

The CREC has been documented in different sectors mainly healthcare settings, livestock, food products, and environment from all over the world (Köck et al. 2018; Cherak et al. 2021). The presence of CREC in aquatic environment raises health concerns as it can be a source for the possible dissemination of the ARGs to the bacterial community across the sectors. The use of contaminated water in aquaculture farms may facilitate the entry or emergence of carbapenem resistance in aquaculture settings and farmed fish, which may be further transmitted to the humans through the food chain or to persons involved in aquaculture activities and fish handling in the retail markets. Therefore, the present work aimed to isolate and characterize the CREC from fish collected from freshwater aquaculture environment and retail fish markets. A key finding of this study is the contamination of CREC in freshwater fish at a low frequency (~7%). The low frequency of CREC has also been reported in previous studies from seafood sold in the retail fish markets from India (Singh et al. 2016). Higher frequencies (> 50%) of CREC have been reported in clinical settings (Manohar et al. 2017; Kumar et al. 2022b) as compared to 27% in aquatic environment receiving hospital effluents (Kalasseril et al. 2020) and 16.5% in livestock (Pruthvishree et al. 2017). The low frequency of CREC in freshwater aquaculture environment can be attributed mainly to the non-use of carbapenems as therapeutic agents in aquaculture. Another noteworthy finding of this study was that the frequency of CREC contamination was significantly higher in fish collected from retail fish markets than directly from the aquaculture environment. This might be due to cross-contamination of the fish in the retail market through contaminated water, ice, storage tanks, improper fish handling, poor hygiene, etc. (Wattimena et al. 2021). The CREC in this study was MDR showing resistance to

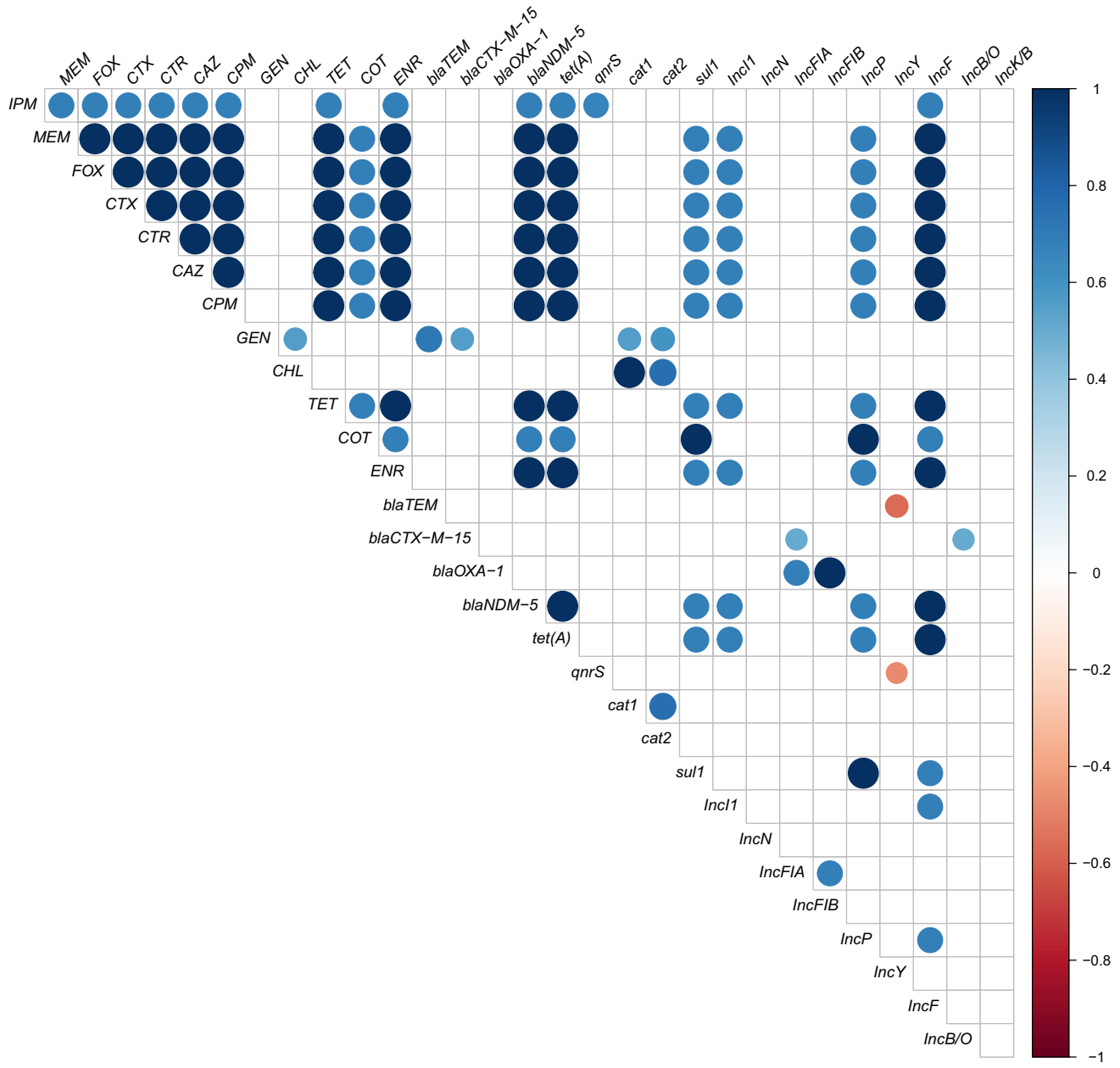

Fig. 3 Correlation matrix illustrating the significant correlation (phi coefficient) between antimicrobial resistance phenotypes (IPM, imipenem; MEM, meropenem; FOX, cefoxitin; CTX, cefotaxime; CTR, ceftriaxone; CAZ, ceftazidime; CPM, cefepime; GEN, gentamicin; CHL, chloramphenicol; TET, tetracycline; COT, co-trimoxazole; ENR, enrofloxacin), antimicrobial resistance genes (ARGs), and incompatible plasmid replicons of carbapenem resistant *E. coli* (CREC). Blue and the red circles show significant ($p < 0.05$) positive and negative correlations, respectively. The intensity of color and the size of the circle represent the strength of the phi correlation coefficient. White squares show a non-significant correlation

carbapenems, cephalosporins, quinolone, tetracycline, and co-trimoxazole. The resistance profiles of the isolates from the aquaculture environment and retail fish markets were similar. However, the MIC of meropenem was significantly higher in isolates from markets than in the aquaculture environment. Not surprisingly, CREC is often reported as MDR in previous studies (Singh et al. 2016; Das et al. 2019). A large number of carbapenemase encoding genes have been reported in CREC all over the world (van Duin and Doi 2017). In India, bla_{NDM} and $bla_{\text{OXA-48}}$ like variants ($bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$) are the most widely reported carbapenemase encoding genes in CREC (Khan et al. 2017; Pitout et al. 2019). In addition, bla_{KPC}, bla_{VIM}, and bla_{IMP} have also been detected in isolates of clinical and animal

origin (Garg et al. 2019; Murugan et al. 2019; Kumar et al. 2022b). Out of the 6 carbapenemase encoding genes screened in this study, we only detected $bla_{\text{NDM-5}}$ in all the isolates supporting the widespread dissemination of NDM producing *E. coli* in India (Khan et al. 2017). Furthermore, $bla_{\text{NDM-5}}$ sequences of CREC in this study were similar to the $bla_{\text{NDM-5}}$ sequences reported from human, livestock, and environmental isolates of CREC from different geographical regions (Hornsey et al. 2011; Aung et al. 2018; Soliman et al. 2020; Das et al. 2019). This finding highlights the possible dissemination of $bla_{\text{NDM-5}}$ between bacterial flora of human, animal, and environment interface and also supports the global dessimination of $bla_{\text{NDM-5}}$ contributing to carbapenem resistance in *E. coli* (Khan et al. 2017).

Although the CREC were resistant to cephalosporins, however, β-lactamase genes responsible for cephalosporin resistance, i.e., bla_{TEM}, $bla_{\text{CTX-M-15}}$, and $bla_{\text{OXA-1}}$ were detected only in 70.6% of the isolates. This signifies that cephalosporin resistance is not mediated alone by these genes. We also observed that cephalosporin resistance phenotypes were significantly associated with $bla_{\text{NDM-5}}$, instead of bla_{TEM}, $bla_{\text{CTX-M-15}}$, and $bla_{\text{OXA-1}}$. So, it can be inferred that the cephalosporin resistance in CREC may be due to the broad hydrolytic activity of $bla_{\text{NDM-5}}$. Hornsey et al. (2011) have previously stated that NDM-5 has a broad hydrolytic activity as compared to other carbapenemases. The β-lactamase genes detected in CREC (bla_{TEM}, $bla_{\text{CTX-M-15}}$, and $bla_{\text{OXA-1}}$) largely encode for ESBL production in Gram-negative bacteria (Queenan and Bush 2007). Surprisingly, none of the CREC was ESBL producers. A possible explanation for this anomaly might be related to the production of NDM-5, which masks the expression of ESBL (Hu et al. 2016). We also found a significant positive association of $bla_{\text{NDM-5}}$ gene with *tet(A)* and *sul1* genes in CREC. Previous studies have also reported the presence of *tet(A)*, *sul1*, and *qnrS* in NDM-5 producing *E. coli* (Liang et al. 2014; Bandyopadhyay et al. 2021). Additionally, a perfectly positive association ($r=1$; $p<0.05$) of *tet(A)* and *sul1* genes to their corresponding resistant phenotypes indicates that the CREC of freshwater aquaculture environment in this study has a very high carriage rate of *tet(A)* and *sul1* genes. The previous study also reported highly transferable *tet(A)* and *sul1* in tetracycline and sulphonamide resistant bacteria of aquaculture origin (Preena et al. 2020). In contrast, complete association ($r=0.454$; $p>0.05$) of *qnrS* gene was not observed in enrofloxacin resistant isolates. This indicates that there might be some other mechanism of quinolone resistance besides *qnrS* gene in CREC, which needs to be explored. Broadly, it can be inferred from our study that the CREC of freshwater aquaculture environment harbors several ARGs that confer multi-drug resistance.

The clonal relatedness study of CREC by ERIC-PCR grouped the isolates into five clusters (I-V). The isolates of clusters I, II, and III was identified as ST167 and cluster IV as ST361, irrespective of the source of isolation. Both these STs are the clonal lineages reported from humans and livestock. In the last few years, high risk clone ST167 and ST361 have been recognized to play an important role in the dissemination of $bla_{\text{NDM-5}}$ all over the world including India (Huang et al. 2016; Dadashi et al. 2019). The sources of dissemination are mainly clinical (Dadashi et al. 2019) and terrestrial animals (Peterhans et al. 2018; Tsilipounidaki et al. 2022). There are few reports on the presence of ST167 and ST361 in aquatic environment (Cherak et al. 2021). To the best of our knowledge, this study is the first report documenting the presence of $bla_{\text{NDM-5}}$ producing *E. coli* ST167 and ST361 from fish of freshwater aquaculture environment in India. The other STs (410, 405, 131, and 101) associated with $bla_{\text{NDM-5}}$, previously reported from clinical settings in India (Devanga Ragupathi et al. 2020; Paul et al. 2020) were not detected in our study. One isolate (CREC-17) that showed a unique genetic pattern did not match with any known STs in the MLST database and this may be a new ST. Although further genetic characterization is necessary before confirming it as a new ST. Strikingly, the CREC in our study belonged to the phylogroup C, which is in contrast to other studies that assigned *E. coli* ST167 and 361 to phylogroup A (Paul et al. 2020). The reason for this anomaly may be that these studies have used triplex PCR for phylogrouping according to Clermont et al. (2000), while we have used the revised phylogrouping method (Clermont et al. 2013). In the revised phylogrouping method, a new phylogroup C has been added which falls between phylogroup A and B1 (closely related to B1). Recently, Chakraborty et al. (2021) also identified *E. coli* ST167 and ST361 into phylogroup C by revised phylogrouping method. Studies have shown that *E. coli* belonging to phylogroup C are multi-drug resistant and less virulent (Denamur et al. 2021). In our study, the commonly reported virulence genes of *E. coli* were not detected in the CREC except for adhesion related genes, *mat* and *fim*C. These two genes facilitate bacterial adhesion and colonization into host cells and transmission of *E. coli* from one host to another (Klemmet al. 2010). This indicates that the CREC of freshwater aquaculture environment has the potential to enter and colonize host cells. So, it is possible that CREC can act as a reservoir for the dissemination of carbapenem resistance from the freshwater aquaculture environment.

Multiple plasmid replicons, namely, IncF, IncFIA, IncFIB, IncI, IncP, IncK/B, IncB/O, IncY, and IncN were detected in the CREC. In this study, a complete association of $bla_{\text{NDM-5}}$ was observed with IncF and strong positive association with IncI1 and IncP. We, therefore, hypothesize the presence of $bla_{\text{NDM-5}}$ and associated ARGs on these transferable plasmids in the CREC. Globally, IncF is the most frequently reported plasmid replicon in NDM-5 producing *E. coli* (Hornsey et al. 2011; Kopotsa et al. 2019;

Chakraborty et al. 2021). Many publications from India have also documented the presence of IncF and its variants in NDM-5 producing *E. coli* isolated from humans (Devanga Ragupathi et al. 2020; Chowdhury et al. 2022), companion animals (Bandyopadhyay et al. 2021), and rivers and sewage treatment plants (Cherak et al. 2021). The plasmid containing IncF replicon has been identified as stable and highly transferrable, which helps in the dissemination of carbapenem resistance among Enterobacteriaceae (Kopotsa et al. 2019). The results of the broth mating experiment in our study confirmed the conjugal transfer of the $bla_{\text{NDM-5}}$ and associated ARGs from CREC to recipient *E. coli* J53. Previously, Chakraborty et al. (2021) have also demonstrated the conjugal transfer of $bla_{\text{NDM-5}}$ through IncF plasmid in *E. coli.* Hence, our study highlights the potential of CREC in plasmid mediated dissemination of NDM-5 and associated ARGs in the aquaculture environment.

Conclusion

This study reports the contamination of carbapenem resistant *E. coli* (ST167 and ST361) harboring $bla_{\text{NDM-5}}$ and associated ARGs in fish of freshwater aquaculture environment at a low frequency, despite the non-use of carbapenems as a therapeutic agent in aquaculture and livestock. This surmises the contamination of the aquaculture environment with carbapenem-resistant bacteria and/or antimicrobials from clinical sources. Moreover, plasmid mediated conjugal transfer of $bla_{\text{NDM-5}}$ and associated ARGs to the susceptible strain also draws attention toward the potential ability of CREC to disseminate antimicrobial resistance, which has health implications and environmental concerns. There is a need for active surveillance of CREC in the aquatic environment for managing the risk of AMR.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-023-25639-9.

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Author contribution The manuscript was reviewed and approved for publication by all authors. Dr. Gaurav Rathore, Dr. Abhishek Awasthi, and Arti Dwivedi contributed to the conception and design of the study. Sampling was done by Dr. Chandra Bhushan Kumar, Anil Kumar, Mayank Soni, Dr. Vikash Sahu, and Arti Dwivedi. Draft manuscript preparation was performed by Arti Dwivedi. The statistical data analysis and manuscript editing was done by Dr. Gaurav Rathore and Dr. Chandra Bhushan Kumar.

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Data availability Nucleotide sequences data generated in this study have been submitted to the NCBI database. The data generated in the study will be provided on request.

Declarations

Ethics approval The field sample collection was done following the animal ethics guidelines of CPCSEA, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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