Molecular assessment of antimicrobial resistance and virulence in multi drug resistant ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* from food fishes, Assam, India

G.K. Sivaraman, S. Shudha, K.H. Muneeb, Bibek Shome, Mark Holmes, Jennifer Cole

Highlights

- CTX-M group 1 (CTX-*M*-15), was the dominant Extended-Spectrum Beta Lactamase (ESBL) found in *E. coli* (98%) and *K. pneumoniae* (100%) recovered from food fishes in India.
- The majority of *E. coli* were assigned to low-virulence phylogroup B1 (40%) and A (30%) while 8% of them belonged to pathogenic phylogroup D.
- Each *E. coli* isolate carried 3 to 5 antimicrobial resistance genes (ARGs) (*tetA*, *dfrA1*, *sul1, sul2, qnrB, qnrS, aac(6')-Ib-cr*).
- *K. pneumoniae* isolates were highly diverse with 11 unique PFGE profiles and a substantial ARG profile (bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1\text{-}like}$, *tetA, strA, strB, dfrA1, sul1, sul2, qnrB, qnrS, aac(6')-Ib-cr, oqxA, oqxB*).
- Co-existence of ESBL genes with other ARGs such as quinolones resistance *(qnrS* and *qnrB)*, tetracycline resistance *(tetA)*, fluoroquinolones *(oqxB* and *oqxA)*, sulphonamides-trimethoprim *(sul2 and dfrA1)* and streptomycin resistance genes (*strA* and *strB*).
- *K. pneumoniae* belonged to capsular serotype (*wzi*gene) and harboured virulence genes *iutA*, *ybtS* and *kfu.*

Abstract

The present study investigated the prevalence of Extended-Spectrum Beta Lactamase (ESBL) -producing *E. coli* and *K. pneumoniae* from the food fishes in retail markets in Assam, India. A total of 54 ESBL-producing *E. coli* and 12 *K. pneumoniae* isolates were recovered from 79 fish samples and were analyzed for antimicrobial resistance genes (ARGs) and virulence genes. *E. coli* isolates were categorized as multi drug resistant with resistance up to 12 different antibiotics with multiple antibiotic resistances (MAR) index ranging from 0.26 to 0.63. In *E. coli*,100% resistance to cefotaxime along with 6% resistance to ceftazidime (third-generation cephalosporins) was observed. Moreover, 85% of the *E. coli* isolates were

resistant to cefepime, a fourth-generation cephalosporin. *K. pneumoniae* showed resistance to 11 different antibiotics with MAR index value ranging from 0.21 to 0.57. All *K. pneumoniae* isolates showed 100% resistance to cefotaxime, 67% resistance to ceftazidime and 75% resistance to cefepime. Molecular characterization of ARGs revealed the presence of CTX-M group 1(CTX-*M*-15) in almost all *E. coli* isolates (98%, $n = 53$) and 100% in *K. pneumoniae*. A combination of uniplex and multiplex PCRs revealed fewer ARGs in *E. coli* isolates, with each isolate carrying 3 to 5 genes (*tetA, dfrA1, sul1, sul2, qnrB, qnrS, aac(6')-Ib-cr*). Majority of the *E. coli* were assigned to low-virulence phylogroup B1 and A while 8% of them belonged to pathogenic phylogroup D. 31 unique genetic profiles were identified for *E. coli* isolates by Pulsed-Field Gel Electrophoresis (PFGE) typing. *K. pneumoniae* isolates were highly diverse with 11 unique genetic profiles and a substantial ARG profile (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA\text{-}1\text{-}like}$, *tetA, strA, strB, dfrA1, sul1, sul2, qnrB, qnrS, aac(6')-Ib-cr, oqxA, oqxB*). The frequency of ARGs ranged between 4 and 11. All *K. pneumoniae* isolates belonged to capsular serotype with *wzi* gene. Virulence gene *iutA* was prominent in all isolates while *ybtS* and *kfu* were confirmed in two isolates. Our findings raise concerns that fishes bought for consumption may serve as potential reservoirs of AMR genes and pose serious threat to public health. The study emphasizes the need for extensive surveillance of resistant strains in aquaculture and related settings, their in-depth analysis of population structure and transmission dynamics.

Keywords: ESBL; MDR; CTX-*M*-15; ARGs; Virulence genes; Phylogroup; PFGE

1. Introduction

Multi drug-resistance (MDR) refers to the non-susceptible microbial isolates to at least one agent in ≥3 antimicrobial categories [1]. The low and middle income countries have prioritized high protein diet in recent times. This transition has facilitated intensive farming by therapeutic use of antibiotics in livestock and agriculture contributing to increased antimicrobial resistance in bacteria [2]. Antibiotics are generally used as growth promoters, prophylactics and for treatment of infections in aquaculture and veterinary sector and these have compounded the risk of transferring resistant bacteria to humans [3]. Antimicrobial resistance is a global issue as exemplified by the rapid increase in cephalosporin and carbapenem resistance in Enterobacteriaceae, leading to a further limitation of treatment options for infections caused by these bacteria [4]. This has triggered the re-introduction of an older and less user-friendly antibiotic, namely colistin, as a last-resort drug for the treatment of critical infections caused by MDR Gram-negative pathogens worldwide [5].

Water bodies remain one of the major reservoirs of MDR bacteria as there is an influx of untreated sewage water, industrial effluents and medical waste from hospital settings. Lack of epidemiological data connecting environmental, industrial and human sectors in terms of dissemination of MDR necessitates surveillance [6]. The state of Assam with an area of 78, 438 km^2 is located between 21.570 N–29.30° N and 89.460 E − 97.30° E. The gigantic Brahmaputra and the Barak rivers with its tributaries adorn the north eastern state of India, giving Assam immense water resources with over 2.86 lakh ha in terms of ponds, tanks, floodplain wetlands called beels and other water bodies. Fish production in the state has risen significantly through the years accounting to 3.07 lakh million tonnes in the year 2016–2017 [7]. Assam is predominantly a fish-eating state (90%–95%) and the per capita consumption is reported to be 9.00 kg [8]. Although the state is endowed with abundant water bodies, it continues to reel from shortfall of fish supply. Though the present production is 3.07 lakh tonnes, it is insufficient to meet the demand of 3.36 lakh tonnes a year. To achieve the gap, Assam still depends on other leading fish producing states in India mainly Andhra Pradesh [9]. Kamrup district is one among the 33 districts of Assam, situated between 25°46' & 26°49′ N and 90°48'& 91°50′ E. The district has a total geographical area of 4345 km^2 and is endowed with good numbers of fisheries resources. The total fish production in the district stands at 22,150 tonnes during 2014–15 and was the third major fish producing district of the state. Besides, the total fish seed production in the Kamrup district is to the tune of 127.65 million numbers during 2014–15. One of the major sources of fishes in this district is capture fishes caught from the mighty Brahmaputra and beels. Aquaculture is promoted in recent times through culturing in ponds and tanks [10,11]. Hazo and Bezera development blocks are the major fish farming regions of Kamrup district. The selected study sites belong to North Guwahati Development Block and were chosen for the antimicrobial resistance (AMR) surveillance study based on several factors. The proximity of the study sites to several hospitals, industrial establishments, small scale poultry farms, domestic animal husbandry units may be a potential source of AMR contamination.

Enterobacteriaceae, the group of bacteria that are normally found in the soft tissues and gastro-intestinal tract of healthy human and other animals are clinically significant as they are carriers of antimicrobial resistance genes (ARGs). The impact of drug resistant bacterial transmission from livestock and aquaculture resources/seafood to humans is poorly understood. Fishes being a major source of food in a state like Assam, it is impending to conduct a surveillance to estimate the prevalence of antimicrobial resistance (AMR) and determine the extent of pathogenicity. Extended-Spectrum Beta Lactamase (ESBLs) are

considered as a major source of resistance in Enterobacteriaceae towards oxyimino-cephalosporins [12]. During 1990s, most ESBLs were TEM or SHV enzyme mutants; from the past decade there has been a surge in CTX-M type ESBLs worldwide. In recent times O157:H7 is one of the prominent Shiga toxin-producing *Escherichia coli* (STEC) responsible for several outbreaks of infection. Non-O157 serogroups belonging to six O groups, O26, O111, O103, O121, O145, and O145 have also been isolated from various food sources and considered as a public health risk [13]. Though there are several reports of AMR and virulence genes identified in *E. coli* isolates collected from river water, surface water, waste/sewage water or sediments or other environmental samples [14, 15, 16] lack of comprehensive data to differentiate between the clinical strains and environmental strains is evident. Reports of virulence genes in domesticated or companion animals are many [17, 18, 19, 20] while there are no substantial reports on *E. coli* strains from fish and fish products with respect to phylogroup, serogroup, virulence/pathogenicity. PCR-based phylogrouping method [21, 22] is considered as one of the robust method to identify pathogenicity of *E. coli* strains irrespective of the source, climate or geography. Shiga toxin-encoding *stx1* and *stx2* are the crucial virulence genes present in shiga toxin producing *E. coli* (STEC) strains. Other virulence factors of *E. coli* O157: H7 and non-O157 STEC include mainly *eae* (encoding the intimin protein), *hlyA* (a plasmid-encoded hemolysin) and *saa* (STEC agglutinating adhesion) [23]. *Escherichia coli* serogroup O2 strains cause extra-intestinal infections in chickens, turkeys, and other avian species [24]. Enteroinvasive *E. coli* (EIEC) strains isolated from milk, beef and cheese have also been linked to diarrheal outbreaks. EIEC O28ac serogroup and STEC O28ac strains are identified from clinical isolates and EIEC serogroup O28ac are reported to be involved in diarrhoea in children [25]. Screening for these virulence genes in isolates recovered from fish will provide an insight to the nature of pathogenicity.

Klebsiella pneumoniae is an important food borne pathogen that can cause septicaemia, liver abscesses and diarrhoea in humans. This Gram-negative Enterobacteriaceae, though a well-known hospital acquired pathogen, is most frequently found in raw vegetables, baby food, fish and meat [26, 27, 28, 29]. Studies on molecular characterization of *K. pneumoniae* from fishes in terms of antibiotic resistance genes, virulence factors and genotyping are scarce. A variety of virulence factors such as the capsules, endotoxins, siderophores, iron-scavenging systems and adhesins play a key role in attributing pathogenicity to the organism. Among the capsular type *K. pneumoniae*, K1 and K2 are known to be highly virulent and have the capability to cause severe infections in humans and animals. Capsular typing and screening

for virulence factors in *K. pneumoniae* by targeting specific genes through PCR has been widely accepted as it is reproducible and enables differentiation in clinical isolates. Detection of virulence factors helps in understanding the nature of the isolate and its pathogenicity thereby enhancing our knowledge in mitigating the threat posed by these organisms. Food being one of the primary routes for antimicrobial resistant bacteria into human gut and growing evidence of certain food items influencing the gut bacterial diversity call for stringent screening of antimicrobial gene determinants [30]. Though K1/K2 are considered as virulent among the capsular *K. pneumoniae,* clinical evidence suggests the incidence of virulence occurring even from some non K1/K2 serotypes. Serotypes that are most frequently encountered in community acquired bacteremia and liver abscess isolates are that of K1, K2, K5, K20, K54 and K57 [31]. It is understood that two genes *magA* (mucoviscosity-associated gene A) and *rmpA* (regulator of mucoid phenotype A) are initially associated with invasive infections. *MagA* is located within the gene cluster of capsular serotype K1 encoding Wzy_{KPK1} and *rmpA* is associated with hyper mucoviscous phenotype [32, 33]. Hypervirulent strains are known to harbour a combination of virulence genes such as enterobactin (*ent*), aerobactin, hydroxamate siderophore whose receptor is encoded by *iutA*, yersiniabactin (*YbtS*), *kfu* – responsible for an iron uptake system (highly prevalent) and *allS*-associated with allantoin metabolism correlate highly with *K. pneumoniae*isolates from liver abscesses particularly with septic endopthalmitis [34, 35]. *Wzi* gene, a conserved gene that encodes an outer membrane protein involved in capsule attachment to the cell surface is present in almost all capsular genotypes of *K. pneumoniae* [31].

The objectives of the present study were to document the prevalence of ESBL, MDR, pathogenicity and virulence profile of the two most important members of Enterobacteriaceae *E. coli* and *K. pneumoniae.* Molecular typing by pulse filed gel electrophoresis (PFGE) and phylogrouping of the ESBL isolates derived from most commonly available edible fishes in the region has been attempted. Reports of incidence rates of AMR and virulence potential of *E. coli* and *K. pneumoniae* isolated from food-fishes is very limited. Extensive surveillance and monitoring to understand the scale of AMR in this region will help to devise antimicrobial stewardship policies at the root level [36].

2. Materials and methods

2.1. Study sites and sample collection

Three sites *viz.,* Silagrant (site 1), Garchuk (site 2) and North Guwahati Town Committee (site 3) were selected in Guwahati, the capital city of Assam state for the collection of fish samples. The chosen study sites had close proximity to different institutional/business set ups like the hospitals, industries, animal husbandry and poultry farms that can act as a possible source of transmission for antimicrobial resistance (AMR). A total of 79 fish samples-site 1 (*n=17*), site 2 (*n=21*) and site 3 (*n=41*) were collected during August 2019 and were transported to the laboratory in a cold storage box. Samples were processed within 3–4 h after their arrival at the laboratory. The gut portions with muscle tissue of the samples were taken for Enterobacteriaceae isolation. Macerated fish samples were aseptically transferred to modified form of Brilliant Green Bile Lactose Broth, EE broth Mossel (BD Difco, USA) enrichment media (pH 7.2) and were incubated for 18–24 h at 35–37 °C. A loopful of the enriched culture was streaked onto MacConkey agar plates (BD Difco, USA) supplemented with 1 μg/ml cefotaxime (Sigma Aldrich, USA) and incubated for 18–24 h at 35–37 °C. Typical pink colored (dry and mucoid) colonies, indicative of lactose-fermenting characteristics of *E. coli* and *K. pneumoniae* were picked. These colonies were further streaked onto Eosin-Methylene Blue agar (EMB- pH 7.2±0.2.) (BD Difco, USA). Presumptive *E. coli* colonies with typical purple colour with or without dark purple centre or with green metallic sheen and large mucoid colonies without metallic sheen (probable Klebsiella) were picked from the EMB plates and were inoculated on to tryptic soy agar (TSA) (BD Difco, USA) slants for identification and further characterization.

2.2. Bacterial species identification, antibiotic susceptibility testing (AST) and MAR index

Isolates recovered from all the three sites were confirmed to the species level using BD Phoenix™ M50 automated system (BD Diagnostics, USA). Procedures were performed according to manufacturer's instructions. Panels were loaded to the instrument within 30 min of inoculation. Quality controls were also performed using the reference strains *E. coli* ATCC 25922. The ID-AST combo panel used in this study, NMIC/ID55 was able to perform susceptibility testing of Gram-negative bacteria against 19 different antibiotics: amikacin (AN), amoxicillin/clavulanate (AMC), ampicillin (AM), aztreonam (ATM), cefazolin (CZ), cefepime (CPM), cefotaxime (CTX), cefoxitin (FOX), ceftazidime (CAZ), chloramphenicol (C), ciprofloxacin (CIP), gentamicin (GN), imipenem (IPM), levofloxacin (LVX), meropenem (MEM), piperacillin (PIP), pipercillin/tazobactam (TZP), tetracycline (TE) and trimethoprim/sulfamethoxazole (SXT) representing 13 antimicrobial categories. Susceptibility patterns were interpreted strictly adhering to CLSI, 2020 [37]. Multiple

antibiotic resistance (MAR) index for *E. coli* and *K. pneumoniae*was calculated based on the number of antibiotics to which the isolates showed resistance to the total number of antibiotics to which isolates were exposed [38].

2.3. PCR screening for antimicrobial resistance genes (ARGs) in *E. coli* and *K. pneumoniae*

Isolates of *E. coli* and *K. pneumoniae*, alerted as 'ESBL-producers' by BD Phoenix™ M50, were screened for the presence of genes encoding β-lactamases. A combination of uniplex and multiplex PCRs were employed for determining AMR genes considering their AST-SIR interpretation. Three multiplex PCRs: a) $bla_{\text{CTX-M}}$ for the CTX-M groups 1, 2 and 9; b) bla_{TEM}/bla_{SHV}/bla_{OXA-1}-like genes and c) plasmid-mediated AmpC β-lactamase gene (pAmpC) combination with six groups were performed [39]. Other than the primer mentioned in the multiplex PCR for CTX-M group 1, primer for gene variant CTX-*M*-15 was also used in the study [40]. Isolates which showed resistance to cefoxitin were tested for pAmpC mediated CMY-2 gene [41]. Simple uniplex were performed for the rest of the genes: quinolone resistance determinants (*qepA, oqxA, oqxB, qnrA, qnrB, qnrS, aac(6')-Ib-cr*), phenicols (*cmlA, cat 1*), aminoglycosides (*strA, strB, aadA1*, *aphA1-1 A B, aac(3)-IV*), tetracycline (*tetA, tetB, tetG)* and folate pathway inhibitors (*dfrA1*, *sul1*, *sul2)*. The primer sequences used in this particular study were obtained from the following reference papers [17, 42 – 52] screened with necessary modifications. The details of the genes tested, primer sequences, concentration of primers used, annealing temperature and expected amplicon size are given in Supl. Table 1. DNA extraction was performed by conventional phenol-chloroform method [53]. PCR reaction was carried out with a final volume of 25 μL containing 1X JumpStart RedTaq ReadyMix (Sigma Aldrich, USA), primers in the desired volume and concentration and finally 2 μL of the DNA extract. Amplicons were visualized after running in 2% agarose gel containing ethidium bromide (1 μg/ml) at 7 V/cm for 2 h and the image was documented (Syngene, UK). A 100 bp DNA ladder (HiMedia, India) was used for comparing the molecular size.

Table 1. Antimicrobial resistance profile of *E. coli* and *K. pneumoniae*.

Antimicrobial[a]	% resistance (no. of isolates)	
	E. coli (n=54)	K.pneumoniae (*n* = 12)
Amoxicillin-Clavulanate	4 (2)	0
Ampicillin	100 (54)	100 (12)

Antimicrobial[a]	% resistance (no. of isolates)	
	E. coli (n=54)	K.pneumoniae (*n* = 12)
Aztreonam	74 (40)	83 (10)
Cefazolin	100 (54)	100 (12)
Cefepime	85 (46)	75 (9)
Cefotaxime	100 (54)	100 (12)
Cefoxitin	4 (2)	0
Ceftazidime	6 (3)	67 (8)
Chloramphenicol	0	8 (1)
Ciprofloxacin	13 (7)	67 (8)
Gentamicin	0	8 (1)
Levofloxacin	13 (7)	17 (2)
Piperacillin	100 (54)	100 (12)
Tetracycline	19 (10)	42 (5)
Trimethoprim-Sulfamethaxozole	17 (9)	50 (6)

[a] *Based on CLSI_2020_M100.*

2.4. Determination of phylogroup, serogroup and virulence genes in ESBL *E. coli*

Improvized PCR based phylogrouping (A, B1, B2, D and F) of the ESBL *E. coli* isolates were determined by quadruplex PCR method [22, 54]. Further categorization to phylogroup C or E was done wherever required [55, 56] Isolates that were not assigned a phylogroup were confirmed for *E. coli* with *uidA* gene. *RfbE*and *fliC* primers were used specifically to detect O157:H7 strains among the *E. coli* isolates [57, 58]. A multiplex PCR which could detect the major non-O157 serogroups (O26, O111, O103, O121, O145, and O145) based on variation in *wzx* (O-antigen flippase) genes in *E. coli* isolates was performed [59, 60]. STEC and EIEC serotypes and associated virulence genes were targeted by two multiplex PCR. i) Shiga toxin producing O2 *wzx*gene, *stx1*, *stx2*, *hly*, *eae* and *saa* genes [61] and ii) pathogenic serogroup O28ac along with EIEC specific virulence genes genes *ial*, *ipaC* and *ipaH* [62, 63, 64, 65]. A third multiplex with primers specifying LT1, LT2 genes associated with enteropathogenic (EPEC) strains and ST1 genes of enterotoxigenic (ETEC) were screened along with *phoA* primer sequence for the confirmation of *E. coli* [66]. Primer sequences and PCR conditions used for the phylogrouping and virulence genes are given in Supl. Table 2.

2.5. K1/K2 serotyping and detection of virulence genes in *K. pneumoniae*

Wzi gene of the *cps* locus was tested to determine the capsular type of the *K. pneumoniae* isolates by PCR [31]. Twelve *K. pneumoniae* isolates were tested for the most frequently encountered K1 and K2 capsular serotypes along with seven virulence genes (*ybtS*, *mrkD*, *entB*, *rmpA*, *kfu*, *allS*, *iutA* and *magA*). Multiplex PCR was performed with the primer sequences and amplicon sizes as given in Supl. Table 2. PCR was kept with 2 µL of DNA extract in a 25 µL reaction volume consisting of Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany). Amplification conditions that were as follows: initial denaturation at 95 °C for 15 min, followed by 30 cycles at 94°C for 30s, 60°C for 90s, and 72°C for 60 s, and a final extension at 72°C for 10min [35].

2.6. Pulsed-field gel electrophoresis (PFGE)

PFGE restriction analyses of the ESBL -*E. coli* and *K. pneumoniae*isolates were performed as per the standard operating procedure by PulseNet International [67]. 200 µl of overnight cultures were lysed and the intact genomic DNA was embedded in agarose plugs. Plugs containing whole genomic DNA of the *E. coli* or *K. pneumoniae* isolates were digested with *Xba*I (50U/sample, New England Biolabs) at 37 °C for 1.5–2 h. DNA fragments were separated on 1% Megabase agarose gel (Bio-Rad, USA) in 0.5X TBE buffer at 14 °C in a CHEF-Mapper XA device (Bio-Rad, USA). Electrophoresis conditions included a constant voltage of 6 V/cm with a run time of 19 h, pulse time ranging from 6.76 S to 35.38 S and an included angle of 120°. *Salmonella* serotype Branderup strain H9812 was used as the standard molecular size marker. The gels were stained with ethidium bromide for 30 min and after subsequent de-staining with deionized water, were documented (Syngene, UK). The DNA fingerprint of each tested isolate was analyzed in BioNumerics ver 7.6 software package (Applied Maths, Belgium). Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Dice coefficient with an optimization and a band matching tolerance of 1.5%. Genetic relatedness was assessed based on the accepted criteria [68].

3. Results

3.1. Baseline data of the isolates

The fish samples collected from the selected study sites in Assam, India were grouped into three types based on the source of collection and relevant ethnographic data from fishermen

and vendors in these sites. The first category of fishes was procured from wholesale markets situated in Guwahati. Here, the majority of fish stock was procured from Andhra Pradesh, West Bengal and Orissa. The state of Assam, though has a vast area of fishery resources (5.49 lakh hectares) in the form of river, ponds, beels, paddy fields and low lying areas, has not yet achieved the self-sufficiency in terms of fish production. In spite of having a vast area of fishery resources, the state has not yet achieved self-sufficiency in terms of fish production. As a result, 0.25 lakh tons of fish is annually imported from other states like West Bengal, Orissa and Andhra Pradesh [69, 70]. The second category was the cultured fishes from the aquaculture farms located in the neighboring villages of Nagaon and Hajo. The most common food fishes available from these two sources were silver carp (*Hypopthalmichthys molitrix*), rohu (*Labeo rohita*), red belly piranha (*Pygocentrus nattereri*) and catla (*Catla*). As the mighty Brahmaputra adjoins the region along the North Guwahati Committee, river-caught fishes were also sold by local vendors. There is a huge demand for these small indigenous fish varieties and people were willing to pay a very high price for these varieties. Hence, the third category included fish varieties which were either river or beel (lake) caught. Indigenous fish varieties such as singara (*Mystus tengara*), singhi (*Heteropneustes fossilis*), kawoi (*Anabas testudineus*), puthi (*Puntius*spp.), bata (*Labeo bata*), aree (*Sperata seenghala*) were some of the varieties in this category. In the present study, around 20% of the fishes collected belonged to the first category and the majority (80%) were local varieties, either cultured or caught from rivers or beels. The details of the isolates along with the fish species and the source of procurement are given (Supl. Table 3). Based on phenotypic observation, 66 non duplicate isolates from 79 fish samples were cultured in pure form for identification.

3.2. Detection of ESBL-producers, AST profile and MAR index

A total of 66 non duplicate isolates that were subjected to identification in BD Phoenix™ M50 were identified as ESBL producers out of which 54 isolates were *E. coli* and 12 were *K. pneumoniae*. NMIC/ID55 panel has a 'BD Phoenix ESBL screening test' which is based on the growth response to selected second or third generation cephalosporins in the presence or absence of a beta-lactamase inhibitor, clavulanic acid. Site wise categorization: 12 *E. coli* and 3 *K. pneumoniae*from site 1-Silagrant (*n=17*); 15 *E. coli* and 6 *K. pneumoniae* from site-2, Garchuk (*n=21*) and 27 *E. coli* and 3 *K. pneumoniae* isolates from site 3-NGTC (*n=41*). In terms of relative abundance of ESBL isolates, *E. coli* accounted for 70.58% from site 1, 71.42% from site 2 and 65.85% from site 3 while the positivity rate were 17.64%, 28.57% and 7.31% for *K. pneumoniae* from site 1, 2 and 3, respectively. In the present study, the relative abundance of *E. coli* was higher as compared to *K. pneumoniae.*

100% of *E. coli* and *K. pneumoniae* isolates showed resistance to beta-lactam antibiotics such as ampicillin, cefazolin, cefotaxime and piperacillin. One ESBL- *E. coli* isolate, each from site 2 and 3 were resistant to cefoxitin. Resistance level of these strains was 75%, 73% and 74% to aztreonam from all the three sites, respectively. Though 100% of the ESBL- *E. coli* isolates showed resistance to cefotaxime, only 6% of them were resistant to ceftazidime, another third-generation cephalosporin; while 85% of the isolates of *E. coli* showed resistance to fourth-generation, cefepime (Table 1) Site wise resistance to cefepime was 67%, 93% and 89% from site 1, 2 and 3, respectively. *K. pneumoniae* isolates showed 67% (8/12) resistance to third-generation ceftazidime and 75% resistant to fourth-generation cefepime. Around 15% of the ESBL- *E. coli* isolates (8/54) and 25% (3/12) ESBL- *K.pneumoniae* isolates were categorized as susceptible-dose-dependent (SDD) for cefepime.

Only 2 isolates of *E. coli* from site 2 and one from site 3 were resistant to cefoxitin. MIC values showed susceptibility for β-lactam combination agents-amoxicillin/clavunate and piperacillin/tazobactam for all isolates except for two isolates of ESBL- *E. coli* each from site 2 and 3. Among the non β-lactam group of antibiotics, 33% of isolates of both *E. coli* and *K. pneumoniae* showed resistance to ciprofloxacin in site 1 and 11% of *E. coli* and 33% of *K. pneumoniae* in site 3. All ESBL- *K. pneumoniae* isolates from site 2 were resistant to aztreonam, cefepime and ciprofloxacin. 33% and 11% of the ESBL- *E. coli* were resistant to levofloxacin in site 1 and 3, respectively while only 2 *K. pneumoniae* isolates showed resistance to levofloxacin. Tetracycline resistance was significantly less in all three sites for ESBL- *E. coli* accounting to 17%, 33% and 11% in site 1, 2 and 3, respectively. With respect to *K. pneumoniae* isolates 67% from site 2 and 33% from site 3 were resistant to tetracycline. Certain number of *E. coli* isolates from all three sites had resistance to trimethoprim/sulfamethoxazole with resistance rates of 8%, 33% and 11%, while it was 33%, 67% and 33% in *K. pneumoniae,* respectively. All the isolates of *E. coli* and *K. pneumoniae* from all three sites were susceptible to amikacin, chloramphenicol (except one *K. pneumoniae* isolate from site 2), gentamicin (except one *K. pneumoniae* isolate from site 3) and carbapenems (imipenem and meropenem) tested. The complete AST data of the isolates tested is given in Supl. Table 4. The representation of percentage of antibiotic resistant isolates from all three study sites is given in Fig 1.

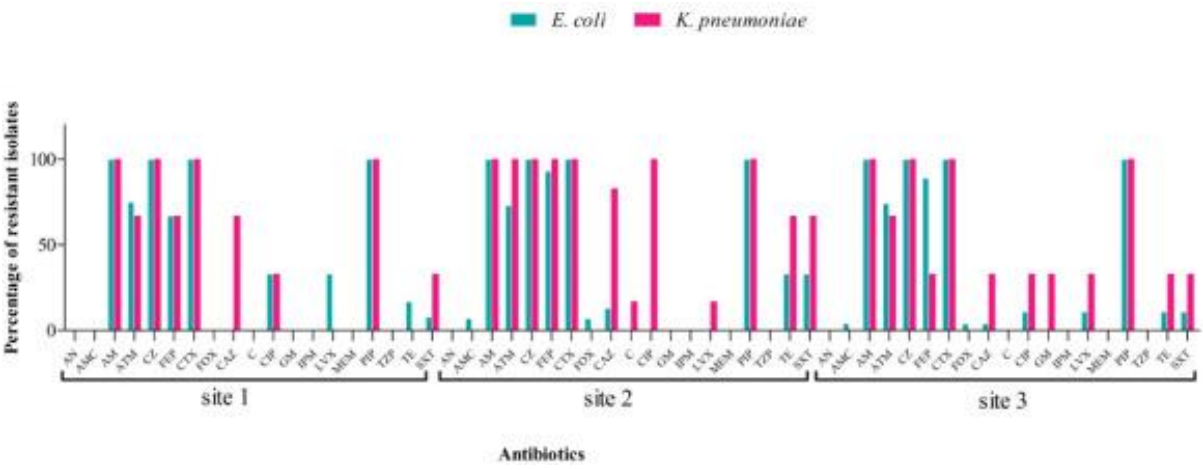


Fig. 1. Resistance pattern of the ESBL-producing E. coli and K. pneumoniae isolates towards different antimicrobials. A comparison between three study sites has been depicted. This is based on the AST report generated by BD Phoenix™ M50 automated system.

MAR index showed a range of 0.26–0.63 in ESBL- *E. coli* isolates with 48% of the isolates with an index value of 0.31. In *K. pneumoniae*, the index value ranged from 0.21 to 0.57 and about 25% of the isolates showed 0.52. This index value of *K. pneumoniae* is inclusive of ampicillin for which *K. pneumoniae* is intrinsically resistant. Both ESBL- *E. coli* and *K. pneumoniae* had showed a higher level of resistance to fourth-generation cephalosporins also. In the present study, all the ESBL isolates showed resistance to more than three antimicrobial categories and hence were considered as multi drug resistant (MDR) [1]. 67% of the *E. coli* and *K. pneumoniae* isolates from site 3-NGTC were found to have resistance to 4 antimicrobial classes. Two *E. coli* isolates from the site 2 and 3 were resistant to a maximum of 8 antimicrobial classes.

3.3. Molecular characterization of ARGs in *E. coli* and *K. pneumoniae*

The most prevalent ESBL determinant in *E. coli* group was CTX-M group 1 (*blaCTX-M-15*) as found in 98% (53/54) of the isolates. Only one isolate from site 3 recovered from *Penaeus* sp. Lacked the CTX-M group 1 gene but showed the presence of plasmid-mediated AmpC-CMY-2 gene. Tetracycline resistance gene, *tetA* was positive in 18% (10) of the isolates none of them carried the other tested *tet* genes (*tetB* and *tetG*). Few ESBL- *E. coli* isolates were affirmative for sulfonamide resistance genes (1 isolate-*sul1*, 2 isolates-*sul2*, 1 isolate-*dfrA1*). Plasmid mediated quinolone gene determinant *qnrS* was present in all *E. coli*isolates irrespective of SIR interpretation (intermediate/resistant) whereas, *qnrB* was detected only in one isolate.

CTX-M group 1 was the dominant β-lactamase gene group with gene variant CTX-*M*-15 (100%) in ESBL-*K. pneumoniae*isolates.83% of the isolates showed co-existence with $bla_{CTX\text{-}M}$and bla_{SHV} gene combination. Resistance gene patterns with respect to TEM were 33% (*n=4*), OXA-17% (*n=2*) and *tetA* was 42% (*n=5*). 8% of the isolates showed aminoglycoside resistant genes *strA, strB,* and sulfonamide resistance genes, *dfrA1* and *sul1*while 42% (*n=5*) of the isolates showed *sul2* gene. *K. pneumoniae* were also screened for the plasmid mediated quinolones antimicrobial (*qnr*) determinants. *QnrB* and *qnrS*were the most detected genes in 92% and 83% of the isolates followed by *oqxB*in 66% (*n=8*) and *oqxA* in 50% (*n=6*). Another quinolone based resistance gene *aac(6')-Ib-cr* was also detected in 50% of the isolates. The diversity of antimicrobial resistance genes shown in ESBL- *K. pneumoniae* isolates from food fishes is given in Table 2. A comparison of the ARG profile with respect to ESBL- *E. coli* and *K. pneumoniae* is given in Fig 2.

Table 2. Distribution of AMR genes in *K. pneumoniae* isolates.

Sl.No.	Isolate ID	Source	Site	AMR genes
1	S1A1	*Pygocentrus nattereri*	1	CTX-*M*-15, qnrS, aac(6′)-Ib-cr
2	S1A6	*Sperata seenghala*	1	CTX-*M*-15,SHV, qnrB, qnrS, aac(6′)-Ib-cr
3	S1A14	*Penaeus* sp.	1	CTX-*M*-15, TEM, SHV, sul2, qnrB, oqxA, oqxB
4	S2A2	*Mystus tengara*	2	CTX-*M*-15, SHV, qnrB, qnrS, oqxA, oqxB
5	S2A9	*Amblypharyngodon mola*	2	CTX-*M*-15, SHV, OXA-1-like, tetA, sul2, qnrB, qnrS, aac(6′)-Ib-cr, oqxB
6	S2A10	*Channa punctatus*	2	CTX-*M*-15, TEM, SHV, tetA, dfrA1, sul1, qnrB, qnrS, oqxA, oqxB
7	S2A16	*Monopterus cuchia*	2	CTX-*M*-15, SHV, qnrB, qnrS, oqxA, oqxB
8	S2A20	*Clarias magur*	2	CTX-*M*-15, TEM, SHV, tetA, sul2, qnrB, qnrS, oqxA, oqxB
9	S2A21	Puria*	2	CTX-*M*-15, SHV, tetA, sul2, qnrB, qnrS, oqxA, oqxB

Sl.No.	Isolate ID	Source	Site	AMR genes
10	S3A10	*Cirrhinus mrigala*	3	CTX-*M*-15, TEM, SHV, OXA-1-like, tetA, sul2, qnrB, aac(6′)-Ib-cr, oqxB
11	S3A12	*Catla*	3	CTX-*M*-15 strA, strB, aadA1, qnrB, qnrS, aac(6′)-Ib-cr
12	S3A16	*Pygocentrus nattereri*	3	CTX-*M*-15, SHV, qnrB, qnrS, aac(6′)-Ib-cr

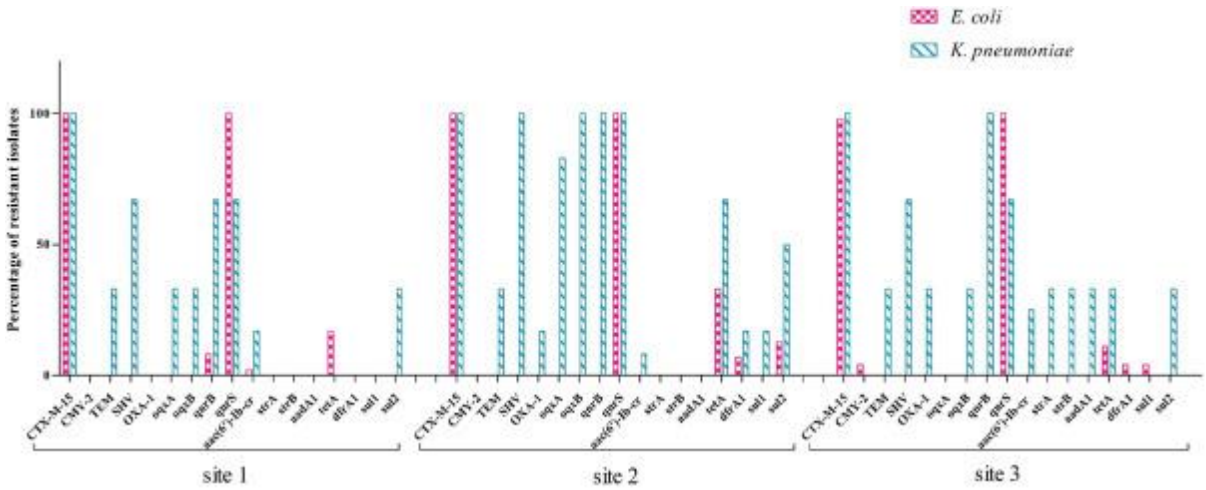


*Fig. 2. Prevalence pattern of resistance genes identified in E. coli and K. pneumoniae from three sites. *local name.*

3.4. Determination of phylogroup, serogroup and virulence genes in *E. coli*

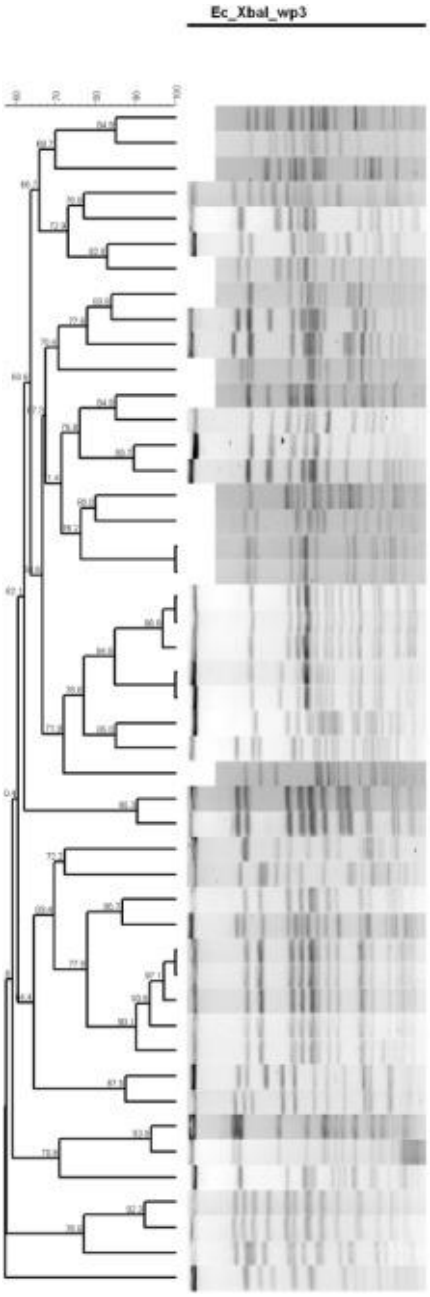
Phylogenetic analysis of the 50 *E. coli* isolates from food fishes collected from three sites showed that 40% (20) of the isolates belonged to phylogroup B1 followed by group A which accounted for 30% (15). Other prominent phylogroups amongst the isolates were E (12%, 6) and D (8%, 4). Two isolates belonged to group F and one isolate to Group C. One isolate could not be typed based on Clermont phylogrouping and was termed ‘Unknown’ as the PCR results were negative for *arpA*/*chuA*/*yjaA*/*TspE4.* This isolate was positive when screened for *E. coli* specific *uidA* gene. None of the isolates belonged to pathogenic B2 phylogroup. 4 isolates could not be retrieved from glycerol stock and hence were not categorized. Among the serogroups tested only 3 *E. coli* isolates belonged to O28ac while none of the isolates belonged to O157:H7 nor the other six major non-O157. Hence we report that ESBL isolates from all three sites do not harbour the virulence genes tested.

3.5. K1/K2 serotyping and detection of virulence genes in *K. pneumoniae*

In the present study, the gene corresponding to capsular serotype K1 and hyper mucoviscosity, *magA* was absent in all the isolates. *RmpA* is known to be prevalent in capsular serotypes K1 and K2 hypervirulent *K. pneumoniae* (hvKp). Absence of *rmpA* gene in the present study indicated that none of the isolates were of K1/K2 serotype. Also the K2 primer targeting the *wzi* gene of the K2 capsular serotype did not amplify in any isolate. Interestingly, *wzi* genes targeting the capsular Klebsiella isolates were all positive for the 12 isolates and hence, the isolates were confirmed as capsular non K1/K2 serotype. *entB*, the most widely spread catecholate siderophore gene and *mrkD,* one of the core genes that occurs in *K. pneumoniae* were confirmed in all the 12 isolates. The 12 non-K1/K2 isolates displayed *iutA* gene while *ybtS* and *kfu* virulence genes were present only in two isolates each. *allS*, the gene encoding allantoin metabolism was absent in all isolates.

3.6. PFGE fingerprint analysis of ESBL-*E. coli* and ESBL-*K. pneumoniae*

Forty seven ESBL- *E. coli* isolates showed 31 distinct PFGE patterns by setting 85% as the similarity cut-off value. ESBL- *E. coli* isolates with 100% similarity occurred in four clusters. The isolates recovered from *Chanda nama* and *Notopterus* were grouped into a single cluster. It was noted that both the fishes were collected from site 2-Garchuk. Similarly, those isolated from *Ompok pabda* and *Labeo rohita* from site 1-Silagrant also showed 100% similarity and was grouped into second cluster. The third cluster comprised isolates recovered from *Penaeus* sp. and *Amblypharyngodon mola* (Silagrant) and these isolated showed indistinguishable PFGE pattern. The isolates in these three clusters belonged to phylogroup A. Two isolates from NGTC site recovered from *Labeo rohita* and *Pangasius* were considered as the fourth cluster with 100% similarity and belonged to phylogroup B1. The similarity between clusters two and three was observed to be 84.86% (Fig 3). Though the isolates in these clusters were from the same location, Silagrant (site 1); other isolates from the same location were grouped in different clusters. On the contrary, none of the ESBL- *K. pneumoniae* showed 100% similarity either within or between the strains from these three sites. Nonetheless, the maximum similarity (88%) observed was among those isolates recovered from fishes, Puria and *Channa punctata (*site 2, Garchuk*)*. In *K. pneumoniae* PFGE genotyping revealed 11 unique lineages (Fig 4).



Isolate ID	Source	Site	Resistance Genes	Phylogroup
S2A2	Mystus tengara	Garchuk	CTX-M-15, dfrA1, sul2, qnrS	B1
S2A11	Labeo rohita	Garchuk	CTX-M-15, qnrS	B1
S2A1	Puntius sophore	Garchuk	CTX-M-15, qnrS	C
S3A38	Puntius serana	NGTC	CTX-M-15, qnrS	A
S3A37	Pseudeutropius atherinoides	NGTC	CTX-M-15, qnrS	E
S1A5	Pungasius pungasius	Silagrant	CTX-M-15, qnrS	B1
S2A15	Lepidocephalus guntea	Garchuk	CTX-M-15, qnrS	E
S2A17	Anabas testudineus	Garchuk	CTX-M-15, qnrS	B1
S3A3	Hypophthalmichthys molit.	NGTC	CTX-M-15, qnrS	B1
S3A23	Ompok bimaculatus	NGTC	CTX-M-15, qnrS	E
S2A18	Pygocentrus nattereri	Garchuk	CTX-M-15, qnrS	B1
S2A21	Puna*	Garchuk	CTX-M-15, qnrS	B1
S3A29	Bulla machi*	NGTC	CTX-M-15, qnrS	B1
S3A30	Karkaria*	NGTC	CTX-M-15, qnrS	B1
S3A24	Labeo gonius	NGTC	CTX-M-15, tetA, qnrS	B1
S2A5	Trichogaster fasciata	Garchuk	CTX-M-15, tetA, qnrS	B1
S2A20	Clarias magur	Garchuk	CTX-M-15, qnrS	Unknown
S2A7	Notopterus notopterus	Garchuk	CTX-M-15, tetA, qnrS	A
S2A8	Chanda nama	Garchuk	CTX-M-15, tetA, qnrS	A
S1A10	Amblypharyngodon mola	Silagrant	CTX-M-15, qnrS	A
S1A14	Penaeus sp.	Silagrant	CTX-M-15, qnrS	A
S1A9	Puntius chola	Silagrant	CTX-M-15, qnrS	A
S1A4	Labeo rohita	Silagrant	CTX-M-15, tetA, qnrB, qnrS	A
S1A13	Ompok pabda	Silagrant	CTX-M-15, qnrS	A
S1A11	Labeo calbasu	Silagrant	CTX-M-15, qnrS	B1
S3A43	Trichogaster fasciata	NGTC	CTX-M-15, qnrS	A
S2A6	Nandus nandus	Garchuk	CTX-M-15, qnrS	A
S3A31	Sperata seenghala	NGTC	CTX-M-15, qnrS	E
S3A32	Mastacembelus armatus	NGTC	CTX-M-15, qnrS	B1
S3A2	Catla catla	NGTC	CTX-M-15, qnrS	D
S3A12	Catla catla	NGTC	CTX-M-15, qnrS	E
S3A42	Channa striatus	NGTC	CTX-M-15, qnrS	B1
S3A28	Penaeus sp.	NGTC	CMY-2, CIT, dfrA1, sul1, qnrS	D
S3A14	Pungasius pungasius	NGTC	CTX-M-15, qnrS	B1
S3A15	Labeo rohita	NGTC	CTX-M-15, qnrS	B1
S3A13	Basunti mas/Bhola*	NGTC	CTX-M-15, qnrS	B1
S3A16	Pygocentrus nattereri	NGTC	CTX-M-15, qnrS	B1
S3A27	Chanda nama	NGTC	CTX-M-15, qnrS	B1
S1A1	Pygocentrus nattereri	Silagrant	CTX-M-15, tetA, qnrS	F
S3A1	Pungasius pungasius	NGTC	CTX-M-15, qnrS	F
S3A35	Channa punctatus	NGTC	CTX-M-15, qnrS	A
S3A33	Narva*	NGTC	CTX-M-15, qnrS	E
S1A12	Cirrhinus mrigala	Silagrant	CTX-M-15, qnrS	A
S3A39	Heteropneustes fossilis	NGTC	CTX-M-15, tetA, qnrS	D
S3A40	Macrognathus aral	NGTC	CTX-M-15, tetA, qnrS	D
S3A41	Anabas testudineus	NGTC	CTX-M-15, qnrS	A
S1A6	Sperata seenghala	Silagrant	CTX-M-15, qnrS	A

Fig. 3. PFGE analysis of E. coli isolates from all three sites.

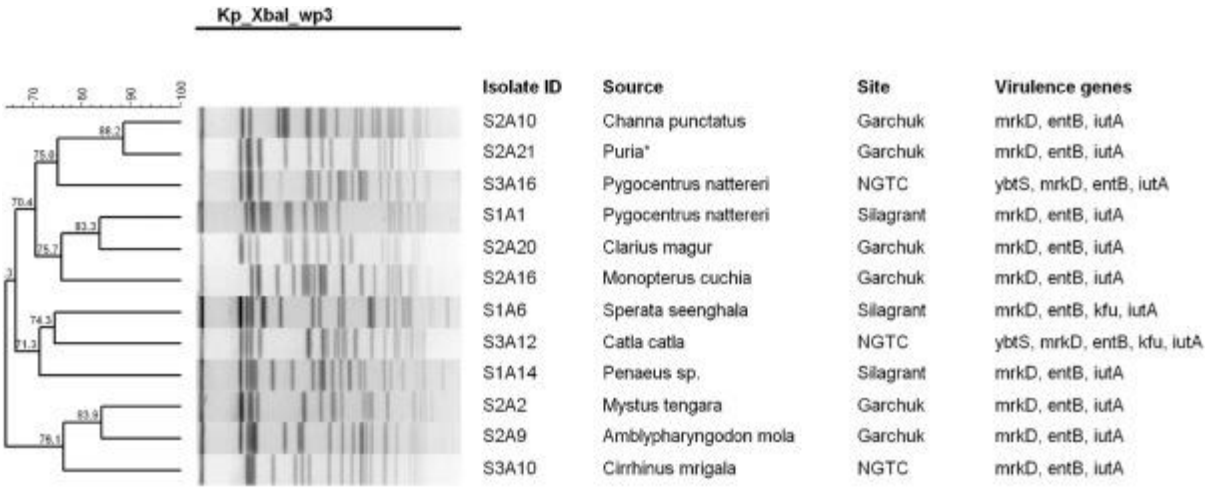


Fig. 4. PFGE analysis of K. pneumoniae isolates from all three sites.

4. Discussions

Fish is one of the main staples in Assam cuisine and 90% of the population consume fish on a daily basis. Certain fish varieties namely *Pangasius, Pygocentrus nattereri, Labeo rohita* and *Catla* studied were generally imported from other states like Andhra Pradesh, West Bengal or Orissa. There is a huge demand for indigenous fish varieties that are locally caught either from river or lakes (beels). In this context, Directorate of Fisheries under the aegis of Government of Assam, India has been promoting aquaculture in villages like Nagaon and Hajo in Kamrup district. In the present study, the fishes collected were from three different localities, but these fishes were either imported from Andhra Pradesh, West Bengal and Orissa, or from local aquaculture sources or captured from local rivers and natural water bodies (beels/lakes). The three selected sites have tertiary hospital settings and industrial establishments in their vicinity which could be a source of contamination through its effluent discharge into these natural water bodies. Irrespective of the fish species, their origin and the site from where it was collected, the study highlights the prevalence of extended spectrum β-lactamase producing *E. coli* (70%) and *K. pneumoniae* (25%) from the fish samples. High incidence of ESBL- *E. coli* (40%) compared to that of *K. pneumoniae* (35%) was reported from 126 wild fish species from Tunisia [71]. Studies on fishes collected from local markets in Tanzania [72] had 2% ESBL producing *E. coli* in them. There are several reports suggesting the persistence of ESBL producing Enterobacteriaceae in livestock and companion animals [73, 74] and even on the surface of the plants, soil, and water [75].

Among the AMR genes studied, CTX-M group-1 was the most dominant in both *E. coli* (CTX-*M*-15, 98%) and *K. pneumoniae*(CTX-*M*-15, 100%). Studies on *E. coli* isolates

from wild fishes from Mediterranean Sea had reported 93.8% of the ESBL- *E. coli*with CTX-*M*-15 gene [76] while CTX-*M*-1 was the major AMR gene prevalent in fishes of lagoons of Tunisia [71]. CTX-M enzymes hydrolyse cefotaxime with little activity against ceftazidime although some of the CTX-*M*-15 had shown strong activity against ceftazidime [77]. This holds true in the present study; although 98% of ESBL *E. coli* have shown CTX-*M*-15 gene, ceftazidime resistance was observed only in three isolates. In terms of *K. pneumoniae*, all of them were CTX-*M*-15 positive and the incidence of ceftazidime resistance MIC was 66% (8/12). Presence of pAmpC mediated CMY-2 was detected in only one *E. coli* isolate from site 3. Studies on freshwater fishes collected from two lakes in Switzerland had reported ESBL- *E. coli* having CTX-M type and one particular isolate with CMY-2 [78]. CTX-M type is known to be one of the most widely circulated AMR genes in the world. CTX-*M*-15 is the most common CTX-M type reported from *E. coli* and *K. pneumoniae* clinical isolates in India [79, 80]. Since they are known to disseminate among the members of the Enterobacteriaceae [81], there is high probability of transmission from aquatic animals to humans and vice versa. Our results confirm high incidence of resistance genes irrespective of the species (fish variety), or geographical location. This correlates with the studies indicating rampant use of antibiotics as prophylactics in aquaculture settings of Andhra Pradesh [82, 83, 84] and also pointed the environmental contamination through domestic sewage, industrial or clinical waste disposal creating high genetic selection pressure for the aquatic microbiota [75, 86, 87, 88]. *E. coli* and *K. pneumoniae* strains along with Citrobacter spp and Entertobacter spp recovered from environmental samples have shown resistance to ciprofloxacin due to anthropogenic contamination [89, 90], but in the present study a significant portion of *E. coli* and *K. pneumoniae* showed either of the quinolone resistance determinants (*qnrB/qnrS/oqxA/oqxB/aac(6')-Ib-cr*) indicating the possibility of environmental contamination. *K. pneumoniae* with relatively lower incidence (25%) was significant in terms of prevalence of diverse AMR genes.

The presence of *E. coli* in environment is considered as a contaminant and any pathogenic strain can be detrimental for human and animal health. *E. coli* found in water or food can be taken as serious threat as it is capable of transferring virulence between and among different bacterial species [91]. In the present study, almost all isolates of *E. coli* screened were categorized into specific phylogroups with the prominent phylogroup being B1 (40%) followed by A (30%). Only one *E. coli*isolate confirmed with *uidA* and *phoA* gene was termed “Unknown” and this could be a rare occurrence. In humans and animals, intra-intestinal infections are caused mostly by phylogroup A/B1 or E and extra-intestinal

infections are caused mainly by strains belonging to B2 [92]. Our findings are in concordance with the reports that *E. coli* strains from animals belong to phylogroup B1 with 34–50% incidence [93, 94, 95]. Similar findings have been reported from the *E. coli* strains isolated from fish samples from Australian waters with B1 as the major phylogroup [96]. 8% of the *E. coli* isolates belonged to phylogroup D which is known to be present in extra-intestinal pathogenic strains and calls for greater alert. Serogroup O28ac usually seen in EIEC strains was detected in two of the *E. coli* isolates belonging to phylogroup B1. Both these isolates were from local fish varieties (puria and bulla machi); any other virulence genes were absent in these isolates. *E. coli* O28ac strains are considered to be human pathogens and are responsible for diarrhoea in children. The presence of these strains in fish samples indicates the possibility of human faecal contamination.

K. pneumoniae can survive in a wide variety of ecological *niche* including soil, water, plant species, insects, birds and mammals. They can exist as free living or host associated. K1 and K2 are capsular serotypes responsible for invasive diseases and enhanced pathogenicity and the most commonly found strain of *K. pneumoniae* in clinical isolates. Capsular production in Klebsiella is attributed to the *Wzy*-dependent process encoded by *wzi, wza, wzb, wzc, wzx* and *wzy* genes of the K-antigen biosynthesis loci. Presence of *wzi* gene in all the isolates indicated that they all belonged to capsular Klebsiella isolates. *Mrk* and *ent* (enterobactin siderophore locus) are part of the core genes of *K. pneumoniae* genome while *ybt* (yersiniabactin locus), *rmp* or any antibiotic resistance genes form the accessory genes which are susceptible for horizontal gene transfer [97]. *MrkD*, a type −3 fimbrial locus is capable of inducing urinary tract infection or cause pneumonia; they can colonize the intestine, form biofilm on catheters [98, 99]. *Ybt* is associated with hospital acquired and also hypervirulent community acquired infections caused by human clinical and colonizing isolates of *K. pneumoniae* [100, 101] and the presence of *ybtS* virulence gene in two of the isolates indicates this possibility. There is a strong correlation between the *kfu* and *allS* with K1 serotype; however in the present study, only two isolates were *kfu* positive while none of the isolates had *allS* or K1 serotype gene targets. It is suggested that *kfu* gene can be present in non K1/K2 isolates [34]. Molecular typing by PFGE showed similarity between isolates among study sites. These findings indicate correlation with the ethnographic data suggesting, irrespective of the geographical location, procurement of the fishes was made from common sources and were distributed into different fish markets enhancing the possibility of ARG transmission.

5. Conclusion

A higher incidence of multi drug resistant β-lactamase-producing *E. coli* and *K. pneumoniae* isolates from the food fish samples collected from Assam, North east India was found in this study. 67% of the isolates were resistant to at least 4 different antimicrobial categories and significant proportions of these isolates were with CTX-*M*-15 type gene. Co-existence of CTX-M, TEM, SHV and plasmid mediated resistance gene determinants especially the quinolone resistance along with tetracycline and sulfonamide resistance genes has been found. Six out of the seven phylogroups (A, B1, C, D, E and F) of *E. coli sensu stricto* were identified. Coexistence of ARGs and virulence factors in *K. pneumoniae* isolates enhance the pathogenicity potential of the isolates. Persistence of ESBL-producing *E. coli* and *K. pneumoniae* underlined the possibility of environmental contamination through sewage, industrial effluent and clinical waste disposal to the natural bodies in the selected study sites. Transmission of ESBL through food chain and environmental contamination has been well documented and the present finding emphasizes the need for greater awareness and hygienic practices among local fishermen and also the community. It is further suggested that antibiotic surveillance program must be ensured by policy makers and appropriate management practices must be implemented in aquaculture settings. AMR stewardship programme may be implemented through campaigns and training programmes at the district and state level for the containment of AMR. Studies including attributes on AMR drivers, virulence factors, and genetic diversity by serogroup, phylogrouping and whole genome sequencing (WGS) will help us to understand the population biology of these pathogens in non-clinical settings. Extensive research on AMR and its transmission to livestock, humans and environment must be fulfilling the greater concept of one health. Genomic analysis of *E. coli* and *K. pneumoniae* isolates from non-human sources is as important as clinical strains in understanding the population and transmission dynamics.

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