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Phenotypic & genotypic study of antimicrobial profile of bacteria isolates from environmental samples

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***Background & objectives*: The resistance to antibiotics in pathogenic bacteria has increased at an alarming rate in recent years due to the indiscriminate use of antibiotics in healthcare, livestock and aquaculture. In this context, it is necessary to monitor the antibiotic resistance patterns of bacteria isolated from the environmental samples. This study was conducted to determine the phenotypic and genotypic profile of antimicrobial resistance in Gram-negative bacteria isolated from environmental samples.**

***Methods*: Two hundred and fifty samples were collected from different sources, *viz.* fish and fishery products (99), livestock wastes (81) and aquaculture systems (70), in and around Mangaluru, India. Isolation, identification and antimicrobial profiling were carried out as per standard protocols. The isolates were screened for the presence of resistance genes using PCR.**

***Results*: A total of 519 Gram-negative bacteria comprising *Escherichia coli* (116), *Salmonella* spp. (14), *Vibrio* spp. (258), *Pseudomonas* spp. (56), *Citrobacter* spp. (26) and *Proteus* spp. (49) were isolated and characterized from 250 samples obtained from different sources. A total of 12 antibiotics were checked for their effectiveness against the isolates. While 31.6 per cent of the isolates were sensitive to all the antibiotics used, 68.4 per cent of the isolates showed resistance to at least one of the antibiotics used. One-third of the isolates showed multidrug resistance. Maximum resistance was observed for ampicillin (43.4%), followed by nitrofurantoin (20.8%). Least resistance was seen for carbapenems and chloramphenicol. PCR profiling of the resistant isolates confirmed the presence of resistance genes corresponding to their antibiotic profile.**

***Interpretation & conclusions*: This study results showed high rate of occurrence of antimicrobial resistance and their determinants in Gram-negative bacteria isolated from different environmental sources.**

Key words Antimicrobial resistance - environmental samples - genotypic characterization - multidrug resistance

The development and spread of antibiotic resistance among bacteria affecting human health are most challenging problems of the modern world. The genetic determinants responsible for conferring

resistance are often carried in self-transmissible mobile elements such as conjugative plasmids, gene cassettes in integrons and transposons, which are transferred between bacterial species, resulting in transmission of resistance to other species[1].

The development of resistance to a specific antimicrobial compound is influenced by several environmental factors and it has been reported that the organisms isolated from environment with high faecal contamination can easily acquire resistance to the common antimicrobial drugs[2]. In addition, the non-therapeutic use of antimicrobials in animal husbandry, aquaculture, agriculture, poultry and piggery has increased the incidence of multidrug resistance forms in the environment[3,4]. The presence of antibiotic resistance in Gram-negative bacteria has been well documented including their occurrence in aquaculture[5] and livestock[6]. Among the Gram-negative bacteria, the prevalence of antimicrobial resistance has been reported to be high in *Escherichia coli*[7], *Salmonella* spp.[8], *Vibrio* spp.[9], *Pseudomonas* spp.[10], *Citrobacter* spp.[11] and *Proteus* spp.[12]. It is important to understand and monitor the antibiotic resistance patterns of human pathogenic bacteria persisting in the environment. Hence, the main objective of this study was to investigate the phenotypic and genotypic profile of antimicrobial resistance in Gram-negative bacteria isolated from environmental samples.

Material & Methods

A total of 250 samples from different sources, *viz*. 99 from fish and fishery products comprising 61 of fish/shellfish and 38 of oyster/clam/molluscs; 81 from livestock wastes comprising poultry (23), piggery (14) and cattle wastes (44) samples and 70 from aquaculture systems comprising fish farm water (40) and pond sediment (30), in and around Mangaluru, India, were aseptically collected fortnightly during the study period (2011-2014). The samples were subjected to isolation of associated bacteria by culture-based conventional methods[13]. Typical colonies from the selective plates were sub-cultured onto Luria Bertani (L-B) agar (HiMedia Laboratories Pvt. Ltd., Mumbai) and identified using standard biochemical tests, *viz*. Gram staining, motility, cytochrome oxidase, catalase, oxidation fermentation test, urease and triple sugar iron agar[14,15]. *E. coli*, *Salmonella* spp. and *Vibrio* spp. were further confirmed by single-step PCR using species-specific primers (Table I)[16-18]. Identified isolates were preserved in 30 per cent glycerol L-B broth and stored at −80°C for further studies.

Antibiotic susceptibility test: Antibiotic susceptibility tests were performed for all the isolates using the disc diffusion method described by Bauer *et al*[19] as per the Clinical and Laboratory Standards Institute guidelines[20]. Twelve common antibiotics (in µg) namely nalidixic acid (30), tetracycline (30), co-trimoxazole (25), ciprofloxacin (5), chloramphenicol (30), ampicillin (10), gentamicin (10), nitrofurantoin (300), imipenem (10), meropenem (10mc), cefotaxime (30) and piperacillin-tazobactam (100/10) (HiMedia) were used for antibiotic profiling. ATCC 25922 *E. coli* culture was used as a standard quality control strain for AST for *Enterobacteriaceae* groups.

Detection of antibiotic resistance determinants by PCR: The isolates showing resistance to particular antibiotics were selected and screened for the presence of antibiotic resistance determinants by PCR using the primers listed in Table II[21-24]. Genomic DNA was extracted from the bacterial culture by cetyl-trimethyl ammonium bromide method[25]. Using genomic DNA as a template, PCR was carried out in 30 µl reaction mixture containing 10× buffer (100 mM Tris-HCl,

Table I. Primers used for the confirmation of suspected *Escherichia coli*, *Salmonella enterica* and *Vibrio* spp. isolates

Isolate	Gene	Sequence 5'- 3'	Size (bp)	Tm (°C)	Reference
Escherichia coli	*uidA*	AAAACGGCAAGAAAAAGCAG	146	60	16
		ACGCGTGGTTACAGTCTTGCG			
Salmonella	*invA*	GTGAAATTATCGCCACGTTCGGGCAA	284	64	17
		TCATCGCACCGTCAAAGGAACC			
	hns	TACCAAAGCTAAACGCGCAGCT	156	60	18
		TGATCAGGAAATCTTCCAGTTGC			
Vibrio	16S rRNA	GTAAATTATCGCCACGTTCGG	321	55	16
		AACGGCAAGAAAAAGCAGTG			

Tm, melting/annealing temperature of primers

Table II. Primers used for detection of different antibiotic resistance genes

Antimicrobials	Resistance genes	Forward and reverse primer 5'-3'	Size (bp)	Present in samples	Reference
Tetracycline	*tetA*	TTGGCATTCTGCATTCACTC	494	Yes	21
		GTATAGCTTGCCGGAAGTCG			
	tetB	CAGTGCTGTTGTTGTCATTAA	571	Yes	
		GCTTGGAATACTGAGTGTTAA			
	tetC	CTTGAGAGCCTTCAACCCAG	418	Yes	
		ATGGTCGTCATCTACCTGCC			
	tetD	GCAAACCATTACGGCATTCT	546	Yes	
		GATAAGCTGCGCGGTAAAAA			
	tetE	TATTAACGGGCTGGCATTTC	544	Yes	
		AGCTGTCAGGTGGGTCAAAC			
	tetG	GCTCGGTGGTATCTCTGCTC	550	Yes	
		CAAAGCCCCTTGCTTGTTAC			
	tetL	CATTTGGTCTTATTGGATCG	488	No	22
		ATTACACTTCCGATTTCGG			
	tetM	GTTAAATAGTGTTCTTGGAG	657	Yes	
		CTAAGATATGGCTCTAACAA			
	tetS	TGGAACGCCAGAGAGGTATT	660	Yes	
		ACATAGACAAGCCGTTGACC			
Sulphonamides	*sul I*	TTTCCTGACCCTGCGCTCTAT	425	Yes	21
		GTGCGGACGTAGTCAGCGCCA			
	sul II	CCTGTTTCGTCCGACACAGA	435	Yes	
		GAAGCGCAGCCGCAATTCAT			
	sul III	ATGAGCAAGATTTTTGGAATCGTAA	792	Yes	
		CTAACCTAGGGCTTTGGATATTT			
Chloramphenicol	*cat1*	AACCAGACCGTTCAGCTGGAT	549	Yes	
		CCTGCCACTCATCGCAGTAC			
	cat2	AACGGCATGATGAACCTGAA	547	Yes	
		ATCCCAATGGCATCGTAAAG			
	cat3	ATCGGCATCGGTTACCATGT	531	No	
		ATCCCCTTCTTGCTGATATT			
	cmlA	GGCCTCGCTCTTACGTCATC	662	Yes	
		GCGACACCAATACCCACTAGC			
	cmlB	ACTCGGCATGGACATGTACT	840	No	
		ACGGACTGCGGAATCCATAG			
	floR	ATGACCACCACACGCCCCG	1,213	No	
		AGACGACTGGCGACTTCTCG			
Quinolones	*qnrA*	ATTTCTCACGCCAGGATTTG	516	Yes	23
		GATCGGCAAAGGTTAGGTCA			
	qnrB	GATCGTGAAAGCCAGAAAGG	469	Yes	
		ACGATGCCTGGTAGTTGTCC			
	qnrS	ACGACATTCGTCAACTGCAA	417	Yes	
		TAAATTGGCACCCTGTAGGC			

Contd...

Antimicrobials	Resistance genes	Forward and reverse primer 5'-3'	Size (bp)	Present in samples	Reference
Ampicillin	bla_{TEM}	CTCACCCAGAAACGCTGGTG	569	Yes	24
		ATCCGCCTCCATCCAGTCTA			
Cefotaxime	bla_{CTX-M}	ACGTTAAACACCGCCATTCC	356	Yes	
		TCGGTGACGATTTTAGCCGC			

*p*H 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin), 200 mM of dNTPs (dATP, dTTP, dGTP and dCTP), 10 pmol each of forward and reverse primers and 1.0 unit of *Taq* DNA polymerase enzyme (HiMedia) in a MJ-Research Thermo Cycler (PTC-200, Bio-Rad, USA). The PCR conditions included initial denaturation at 94°C for 5 min, followed by 35 cycles with each cycle consisting of denaturation at 94°C for 60 sec, annealing for 60 sec at an optimized temperature depending on the primer set used and extension at 72°C for 30 sec. The final extension was set at 72°C for 10 min.

Sequencing of antibiotic-resistant determinants: The amplified PCR products (antibiotic-resistant determinants) were purified using PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and were outsourced for capillary sequencing. The obtained sequences were analyzed by BLAST (*http://blast.ncbi.nlm.nih.gov*) and antibiotic resistance genes database for their homology with the database sequences and the confirmed sequences were submitted to the GenBank.

Results

Five hundred and nineteen bacterial strains were isolated and identified to the genus level by conventional methods using a battery of biochemical tests. The details of the isolates identified from the different sources are given in Table III. Molecular confirmation of *E. coli, Vibrio* spp. and *Salmonella* spp. was done by PCR using primers namely *uidA* for *E. coli, invA* and *hns* for *Salmonella* and 16S rRNA for *Vibrio* (Fig. 1).

Antimicrobial susceptibility test: Among the 519 isolates tested for antimicrobial susceptibility, only 31.6 per cent (164 isolates) showed susceptibility to all the antibiotics tested. While 68.4 per cent (355 isolates) were resistant to at least one of the antibiotics, 33.7 per cent (175 isolates) displayed multidrug resistance (resistance to more than one antibiotic). As shown in Fig. 2, maximum resistance was observed for ampicillin (225 isolates, 43.4%) followed by nitrofurantoin (108 isolates, 20.8%), nalidixic acid (71 isolates, 13.7%), cefotaxime (62 isolates, 11.9%), tetracycline (58 isolates, 11.2%), co-trimoxazole (38 isolates, 7.3%), ciprofloxacin (30 isolates, 5.8%), gentamicin and piperacillin/tazobactam (28 isolates, 5.4%), imipenem (18 isolates, 3.5%), meropenem (16 isolates, 3.1%) and chloramphenicol (15 isolates, 2.9%).

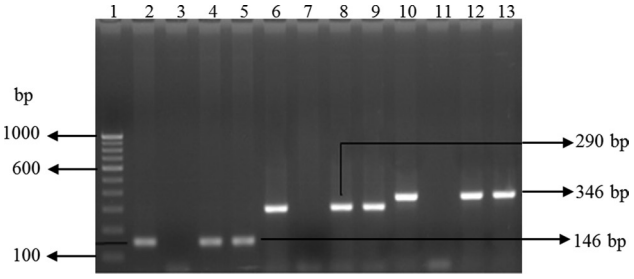

Fig. 1. Gel-electrophoresis of PCR amplified products of *Escherichia coli* and *Salmonella* isolates. Lane1: 100 bp DNA ladder; lane 2: *uidA* gene-positive control (*E. coli*); lane 3: *uidA* gene-negative control (*E. coli*); lanes 4 & 5: *uidA* gene-positive *E. coli* isolates; lane 6: positive control for *invA* gene (*Salmonella*); lane 7: negative control for *invA*; lanes 8 & 9: *invA* gene-positive *Salmonella* isolates; lane 10: positive control for *hns* gene (*Salmonella*); lane 11: negative control for *hns* gene; lanes 12-13: *hns* gene-positive *Salmonella* spp. isolates.

The drug resistance patterns of the isolates according to the source of collection are presented in Fig. 3. Among isolates obtained from fishery products and environment samples, 47.5 per cent showed resistance to ampicillin and 18 per cent to nitrofurantoin, suggesting maximum resistance to these two antibiotics. Among isolates from livestock wastes, maximum resistance was observed for nitrofurantoin (33.6% of the isolates) followed by ampicillin (28.6% of the isolates) and tetracycline (22.7% of the isolates).

Detection of antibiotic resistance genes: The antibiotic-resistant determinants associated with resistance were detected by PCR. Of the 58 isolates showing resistance to tetracycline, 45 (77.6%) harboured one or more than one tetracycline-resistant genes (*tetA, tetB, tetC, tetD, tetE, tetG*, *tetM* and *tetS*). Remaining 13 isolates did not carry any of the *tet* genes tested even though these were phenotypically resistant. Among 38 isolates resistant to co-trimoxazole, 12 (31.6%) harboured at

Table III. Total number of Gram-negative bacterial strains isolated from different non-human sources

Source	Number of samples	*Escherichia coli*	*Vibrio* spp.	*Salmonella* spp.	*Pseudomonas* spp.	*Citrobacter* spp.	*Proteus* spp.	Total
Fish and fishery products (fish/shrimps and clams/oysters/ mussels)	99	45	169	8	18	6	13	259
Livestock wastes (poultry, piggery and cattle farm)	81	61	7	6	17	10	18	119
Environmental samples (farm water and pond sediment)	70	10	82	-	21	10	18	141
Total	250	116	258	14	56	26	49	519

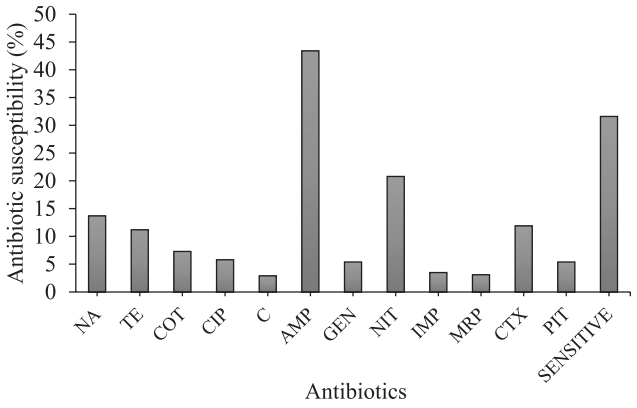

Fig. 2. Antibiotic resistance pattern of all isolates against 12 different antibiotics used in the study. NA, nalidixic acid; TE, tetracycline; COT, co-trimoxazole; CIP, ciprofloxacin; C, chloramphenicol; AMP, ampicillin; GEN, gentamicin; NIT, nitrofurantoin; MRP, meropenem; CTX, cefotaxime; PIT, piperacillin/tazobactum.

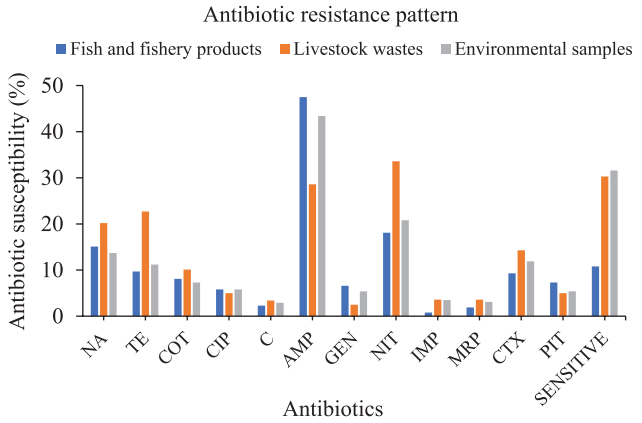

Fig. 3. Source-wise representation of antibiotic resistance pattern of the isolates to 12 antibiotics. Abbreviations are as given in Fig. 2.

least one of the *sul* genes (*sul1, sul2*, *sul3*) and the 26 isolates did not harbour any of the tested genes (Fig. 4). Two hundred and twenty five ampicillin-resistant isolates were tested for the presence of bla_{TEM}, the gene responsible for the resistance. However, only eight isolates (3.6%) showed the presence of this gene. Of the 62 cefotaxime-resistant isolates, only five (8%) had the $bla_{CTX\text{-}M}$ gene conferring the resistance trait. Among the 15 isolates resistant to chloramphenicol, five (33.3%) carried one of the resistance genes (*cat1, cat2* and *cmlA*), but none of these showed the presence of *cat3*, *cmlB* and *floR* (Fig. 5). Of the 71 nalidixic acid-resistant isolates, nine (12.7%) carried either *qnrA, qnrB* or *qnrS*.

Further, sequencing of the PCR products of antibiotic resistance genes from representative isolates revealed 98 per cent identity with the existing antibiotic resistance gene sequences in the database. The GenBank accession numbers of these sequences are given in Table IV.

Discussion

The results of antibiotic susceptibility test revealed that the number of isolates showing resistance to one or more antibiotics was on the rise, suggesting the high occurrence of antibiotic resistance in animal, wastewater, soil and other natural environments. Regardless of their source of isolation, resistance to three antibiotics, namely ampicillin, nitrofurantoin and tetracycline, was most frequently observed. Although ampicillin resistance was highest among all the antibiotics used.

It has been shown that isolates from fish and shrimp farms have widespread resistance to nitrofurantoin[26]. In this study, approximately 20 per cent of the isolates showed resistance to nitrofurantoin. Similarly, 18 per cent of the isolates obtained from fish farms and related products showed resistance to this antibiotic, and the results were in agreement with that of an earlier

study[27]. The resistance to nitrofurantoin could be either due to the inhibition of nitrofurantoin reductase or due to the nucleotide changes (mutation) in *nfs*A and *nfs*B encoding oxygen-insensitive nitro reductase. Nitrofurantoin and its metabolites have zero tolerance in fishery products, and yet the high occurrence of resistance to nitrofurantoin observed in the study indicating the use of this antibiotic in aquaculture and other environments.

Resistance to tetracycline has been reported frequently from environmental samples[28]. In the present study, 11.2 per cent of the isolates exhibited phenotypic resistance to tetracycline, which was consistent with earlier studies that reported tetracycline resistance in 15-56 per cent of the isolates[28]. Resistance to tetracycline is more frequently observed in poultry isolates because it is widely used as growth promoters in poultry production[29]. More than 60 per cent of poultry isolates showed resistance to tetracycline in this study.

Skockova *et al*[30] had earlier reported that *tetA* and *tetB* were the most common genes responsible for resistance to tetracycline. In our study, 77 per cent of the phenotypically resistant isolates showed the presence of one or more *tet* genes. Although the remaining 23 per cent did not carry any of the *tet* genes tested in the study despite being phenotypically resistant, the resistance in such isolates could be due to other mechanisms such as enzymatic inactivation or target modification[31].

In this study, 7.3 per cent of the total isolates showed moderate resistance to co-trimoxazole. The sulphonamide resistance genes (*sul1*, *sul2* and *sul3*) are known to be associated with class 1 integrons responsible for capturing and excision of genes during site-specific recombination events[32], which further facilitates the emergence of multidrug resistance among bacterial pathogens. Similar to the pattern seen for other antibiotics, the phenotypic and genotypic association was inconsistent for co-trimoxazole with only 31.6 per cent of the phenotypically confirmed isolates showing the presence of one of the three *sul* genes. The absence of *sul* in the remaining isolates suggests the possibility of some new genes or resistance mechanism to confer resistance to co-trimoxazole.

The antibiotic resistance pattern obtained for chloramphenicol in this study was low profile *i.e.*,2.9 per cent. The results of genotypic

Table IV. List of antibiotic resistant gene sequences submitted to GenBank with accession number

Title	Accession number
Escherichia coli isolate T1 tetracycline resistance protein class A (*tetA*) gene, partial cds. (EC12)	KF240812.1
E. coli strain T2 tetracycline resistance protein class B (*tetB*) gene, partial cds. (EC23)	KF240811.1
Salmonella sp. T1a tetracycline resistance proteins class A (*tetA*) gene, partial cds. (S131)	KF240813.1
Salmonella sp. S1a sulphonamide resistance protein (*sul1*) gene, partial cds. (S131)	KF240817.1
E. coli strain S2 sulphonamide resistance protein (*sul2*) gene, partial cds. (EC11)	KF240815.1
E. coli strain S3 sulphonamide resistance protein (*sul3*) gene, partial cds. (EC10)	KF240814.1
E. coli strain S1 sulphonamide resistance protein (*sul1*) gene, partial cds. (EC10)	KF240816.1

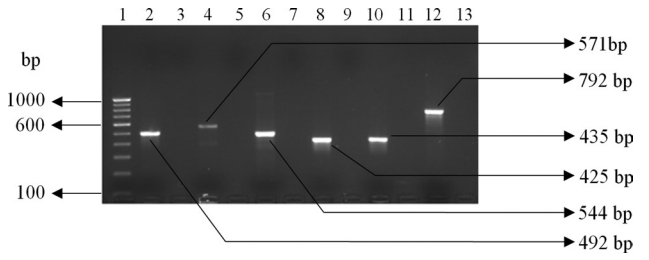

Fig. 4. Detection of PCR amplified products of antibiotic resistance genes. Lane 1: 100 bp DNA ladder; lane 2: *tetA* (492 bp); lane 4: *tetB* (571 bp); lane 6: *tetE* (544 bp); lane 8: *sul1* (425 bp); lane 10: *sul2* (435 bp); lane 12: *sul3* (792 bp); lane 14: 500 bp DNA ladder and lanes 3, 5, 7, 9, 11, 13: negative controls.

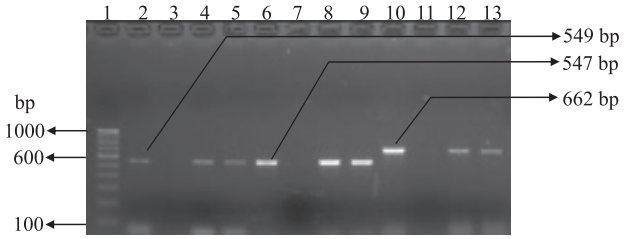

Fig. 5. Gel-electrophoresis of PCR amplified products of chloramphenicol-resistant isolates. Lane 1: 100 bp DNA ladder; lane 2: *cat1* gene-positive control; lane 3: *cat1* gene-negative control; lanes 4-5: *cat1* gene-positive isolates; lane 6: positive control for *cat2* gene; lane 7: negative control for *cat2* gene; lanes 8-9: *cat2* gene-positive isolates; lane 10: positive control for *cmlA* gene; lane 11: negative control for *cmlA* gene; lane 12-13: *cmlA* gene-positive isolates.

characterization showed inconsistency with 20 per cent carrying both type 1 and type 2 *cat* genes and 13.3 per cent harbouring *cmlA*. However, none of the isolates were positive for type 3 *cat*, *cmlB* and *floR*. The absence of resistance genes was observed in 33.3 per cent of the phenotypically resistant isolates, suggesting the possibility of other mechanism(s) such as overexpression of efflux pumps, mutations or modifications in the target sites or decreased outer membrane permeability contributing to chloramphenicol resistance[33,34].

The resistance to β-lactam group of antibiotics such as ampicillin, cefotaxime, imipenem, meropenem and piperacillin/tazobactam was also analyzed in this study. Except for ampicillin, which showed maximum resistance, the percentage of resistance was relatively less with 3.5 and 3.1 per cent of isolates showing resistance to imipenem and meropenem, respectively. In our study, 20 per cent of the isolates showed resistance to nalidixic acid and five per cent towards ciprofloxacin. The usual cause of resistance to quinolones is due to point mutation/mutations in the quinolone resistance determining regions (QRDR) or due to presence of active efflux or outer membrane permeability. In addition, the plasmid-mediated quinolone resistance (PMQR) has also been reported. In the present study, nalidixic acid- and ciprofloxacin-resistant isolates carried *qnrA*, *qnrB* and *qnrS* indicating that quinolone resistance was acquired through plasmid-mediated determinant. Although point mutations in the QRDR are the main reason for resistance to quinolone/fluoroquinolones, the occurrence of PMQR genes cannot be neglected since these play a major role in the transmission of resistance among bacterial isolates[35]. The bacterial isolates showed similar pattern of resistance to cell wall synthesis inhibitors such as meropenem and imipenem. Although the susceptibility of the majority of the isolates to carbapenems was encouraging, the small percentage of resistance observed should be viewed seriously.

In conclusion, the results of this study on the pattern of resistance to antimicrobials in environmental samples highlighted the importance of continuous vigilance on the distribution of multidrug-resistant human pathogens in the environment. High rate of multidrug resistance among bacterial isolates from environmental samples suggested the indiscriminate use of antimicrobials in various sectors of animal husbandry. Judicious use of antibiotics, use of alternative bio-control approaches or development of pathogen-specific antimicrobial agents could help in combating antimicrobial resistance.

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***Conflicts of Interest*:** None.

References

1. Richardson LA. Understanding and overcoming antibiotic resistance. *PLoS Biol* 2017; *15* : e2003775.
2. Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B, *et al.* Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Appl Environ Microbiol* 2009; *75* : 5714-8.
3. Collignon P, Wegener HC, Braam P, Butler CD. The routine use of antibiotics to promote animal growth does little to benefit protein undernutrition in the developing world. *Clin Infect Dis* 2005; *41* : 1007-13.
4. Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: History and mode of action. *Poult Sci* 2005; *84* : 634-43.
5. Sarter S, Nguyen HN, Hung LT, Lazard J, Montet D. Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish. *Food Control* 2007; *18* : 1391-6.
6. Srisanga S, Angkititrakul S, Sringam P, Le Ho PT, Vo AT, Chuanchuen R, *et al.* Phenotypic and genotypic antimicrobial resistance and virulence genes of *Salmonella enterica* isolated from pet dogs and cats. *J Vet Sci* 2017; *18* : 273-81.
7. Sukhumungoon P, Nakaguchi Y, Ingviya N, Pradutkanchana J, Iwade Y, Seto K, *et al*. Investigation of stx 2+ eae+ *Escherichiacoli* O157: H7 in beef imported from Malaysia to Thailand. *Int Food Res J* 2011; *18* : 381-6.
8. Chen S, Zhao S, White DG, Schroeder CM, Lu R, Yang H, *et al.* Characterization of multiple-antimicrobial-resistant *Salmonella serovars* isolated from retail meats. *Appl Environ Microbiol* 2004; *70* : 1-7.
9. Akond MA, Alam S, Hasan SM, Uddin SN, Shirin M. Antibiotic resistance of *Vibrio cholerae* from poultry sources of Dhaka, Bangladesh. *Adv Biol Res* 2008; *2* : 60-7.
10. Yayan J, Ghebremedhin B, Rasche K. Antibiotic resistance of *Pseudomonas aeruginosa* in pneumonia at a Single University Hospital Center in Germany over a 10-year period. *PLoS One* 2015; *10* : e0139836.
11. Khanna A, Singh N, Aggarwal A, Khanna M. The antibiotic resistance pattern in *Citrobacter* species: An emerging nosocomial pathogen in a tertiary care hospital. *J Clin Diagn Res* 2012; *6* : 642-4.
12. Feglo PK, Gbedema SY, Quay SN, Adu-Sarkodie Y, Opoku-Okrah C. Occurrence, species distribution and antibiotic resistance of *Proteus* isolates: A case study at the Komfo Anokye Teaching Hospital (KATH) in Ghana. *Int J Pharm Sci Res* 2010; *1* : 347-52.
13. Andrews WH, Wang H, Jacobson A, Hammack TS. *Salmonella*. In: *Bacteriological analytical manual*. Gaithersburg, MD: Federal Drug Administration; 2011.

14. Khuntia BK. *Basic microbiology: A illustrated laboratory manual*. New Delhi, India: Daya Publishing House; 2011.
15. Mac Faddin JF. *Biochemical tests for identification of medical bacteria*. Philadelphia: Williams & Wilkins Co.; 1976.
16. Bej AK, DiCesare JL, Haff L, Atlas RM. Detection of *Escherichiacoli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for uid. *Appl Environ Microbiol* 1991; *57* : 1013-7.
17. Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galán JE, Ginocchio C, *et al*. Amplification of an *InvA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol Cell Probes* 1992; *6* : 271-9.
18. Jones DD, Law R, Bej AK. Detection of *Salmonella* spp. in oysters using polymerase chain reaction (PCR) and gene probes. *J Food Sci* 1993; *58* : 1191-7.
19. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; *45* : 493-6.
20. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial disk susceptibility tests.* 28thed. CLSI Document M100. Wayne, PA: CLSI; 2018.
21. Ma M, Wang H, Yu Y, Zhang D, Liu S. Detection of antimicrobial resistance genes of pathogenic *Salmonella* from swine with DNA microarray. *J Vet Diagn Invest* 2007; *19* : 161-7.
22. Aarestrup FM, Agerso Y, Gerner-Smidt P, Madsen M, Jensen LB. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis* 2000; *37* : 127-37.
23. Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA, Hooper DC. Qnr prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. *Antimicrob Agents Chemother* 2006; *50* : 2872-4.
24. Colomer-Lluch M, Jofre J, Muniesa M. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS One* 2011; *6* : e17549.
25. Ausubel FM, editor. *Current protocols in molecular biology*. 4th ed. New Jersey: John Wiley & Sons; 1995.
26. Kumar R, Surendran PK. Plasmid-mediated antibiotic resistance in *Pseudomonas* (*Burkholderia*) *cepacia* from brackish water aquaculture farms. *Indian J Fish* 2006; *53*: 263-9.
27. Arredondo-García JL, Amábile-Cuevas CF. High resistance prevalence towards ampicillin, co-trimoxazole and ciprofloxacin, among uropathogenic *Escherichia coli* isolates in Mexico city. *J Infect Dev Ctries* 2008; *2* : 350-3.
28. Chikwendu CI, Ibe SN, Okpokwasili GC. Multiple antimicrobial resistance in *Vibrio* spp. isolated from river and aquaculture water sources in Imo state, Nigeria. *J Br Microbiol Res* 2014; *4* : 560-9.
29. Kolar M, Pantucek R, Bardon J, Vagnerova I, Typovska H, Valka I, *et al*. Occurrence of antibiotic-resistant bacterial strains isolated in poultry. *Vet Med Czech* 2002; *47* : 52-9.
30. Skockova A, Cupakova S, Karpískova R, Janstova B. Detection of tetracycline resistance genes in *Escherichia coli* from raw cow's milk. *J Microbiol Biotechnol Food Sci* 2012; *1* : 777.
31. Chopra I, Roberts M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001; *65* : 232-60.
32. Deekshit VK, Kumar BK, Rai P, Srikumar S, Karunasagar I, Karunasagar I, *et al.* Detection of class 1 integrons in *Salmonella* weltevreden and silent antibiotic resistance genes in some seafood-associated nontyphoidal isolates of salmonella in South-West coast of India. *J Appl Microbiol* 2012; *112* : 1113-22.
33. Daniels C, Ramos JL. Adaptive drug resistance mediated by root-nodulation-cell division efflux pumps. *Clin Microbiol Infect* 2009; *15* (Suppl 1) : 32-6.
34. Montero CI, Johnson MR, Chou CJ, Conners SB, Geouge SG, Tachdjian S, *et al.* Responses of wild-type and resistant strains of the hyperthermophilic bacterium *Thermotoga maritima* to chloramphenicol challenge. *Appl Environ Microbiol* 2007; *73* : 5058-65.
35. Santhosh KS, Deekshit VK, Venugopal MN, Karunasagar I, Karunasagar I. Multiple antimicrobial resistance and novel point mutation in fluoroquinolone-resistant *Escherichiacoli* isolates from Mangalore, India. *Microb Drug Resist* 2017; *23* : 994-1001.

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