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Presence & mobility of antimicrobial resistance in Gram-negative bacteria from environmental samples in coastal Karnataka, India

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To understand antimicrobial resistance (AMR) patterns and mechanisms of horizontal gene transfer in human-associated environments is essential to AMR surveillance. Gram-negative bacteria (1122 isolates) from food-animal environments were characterized for antimicrobial susceptibility and AMR genes. Seventy five per cent of the isolates (837 of 1122) were resistant to at least one of the antibiotics tested. Resistance to more than three groups of antimicrobials (multidrug resistance) was observed in 43 isolates with most often encountered (12 of 43) resistance to β-lactams, tetracycline, quinolones and nitrofurantoin. The profile of frequently reported plasmid-mediated resistance gene in these isolates was determined. The mobility of these elements as plasmids or phages was examined. The *bla*$_{CTX-M}$ gene was present in the plasmid of 61 per cent and packed in induced phage fractions in 72 per cent of the isolates and *bla*$_{TEM}$ in 69 per cent phage fractions compared to 15 per cent presence in the plasmid.

Key words Antimicrobial resistance - environment - mobile genetic elements - prophage induction

Microbial infections are the second leading cause of death worldwide with infections due to Gram-negative bacteria increasing at an alarming rate[1]. The use of antimicrobials does not occur only with regard to human diseases and in hospital environment but is also a part of animal husbandry and agricultural practices[2]. Livestock and agricultural practices include the use of antibiotics for disease prevention and growth promotion of animals. The recurrent use of antibiotics in these environments makes these areas as potential reservoirs of drug-resistant bacteria which can spread to other animals, humans and the environment[3].

The emergence of resistance has revealed mechanisms by which resistance genes spread across the bacterial kingdom, with apparent disregard for species barriers which is facilitated by horizontal gene transfer by mobile genetic elements[2,3]. The transmissibility and plasticity of antimicrobial resistance determinants (ARDs) imply an alarming potential to spread and diversify among bacterial populations with plasmids and phages playing a significant role in this[4-6]. ARDs *bla*$_{TEM,}$ *bla*$_{CTX-M}$, *tet*A, *qnr*A and *qnr*S have been reported in phage DNA fractions in the environment and separate studies have reported these in plasmids[7-11]. Bacterial genes can randomly get packed into phage heads by generalized transduction during infection with a lytic phage. We have reported *bla*$_{CTX-M}$ in lytic bacteriophages of *Escherichia coli* that transfer this ARD via generalized transduction[12].

Thirty samples each of seafood, aquaculture farms (soil and water), poultry farms, piggeries, industrial effluents, domestic effluents and hospital effluents and 32 samples from livestock environments were collected at random (from July 2014 to 2016) from various locations in and around Mangalore, India. The study was conducted in the division of Infectious Diseases, Nitte Centre for Science Education & Research, Mangalore, India. Standard procedures for the isolation of *Vibrio* sps, *Pseudomonas* sps and *Enterobacteriaceae* members *E. coli*, *Klebsiella sps, Enterobacter* sps and *Salmonella* sps, were followed[13-16].

Typical colonies were picked in each case and purified on tryptic soya agar. Isolated pure cultures were characterized by a battery of biochemical test[13-16] and complemented with PCR-based methods for genotypic identification of certain species, namely *E. coli*[17], *Salmonella*[18] and *Vibrio parahaemolyticus*[19]. Antimicrobial susceptibility test was carried out by disk diffusion method for antibiotics representing the major groups such as penicillins [ampicillin (AMP) 10 µg, piperacillin/tazobactam 100/10 µg], cephalosporins [cefotaxime (CTX) 30 µg], aminoglycosides [gentamicin (GEN) 10 µg], quinolones [nalidixic acid (NA) 30 µg, ciprofloxacin (CIP) 5 µg], chloramphenicol (C) 30 µg, tetracycline (TET) 30 µg, nitrofurantoin (NIT) 300 µg, sulphonamides (co-trimoxazole 25 µg) and carbapenems [imipenem (IMP) 10 µg, meropenem (MRP)10 µg] as per Clinical Laboratory Standards International guidelines 2012[20]. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. Antimicrobial discs and control strains were procured from HiMedia Laboratories (Mumbai). PCR-based screening of

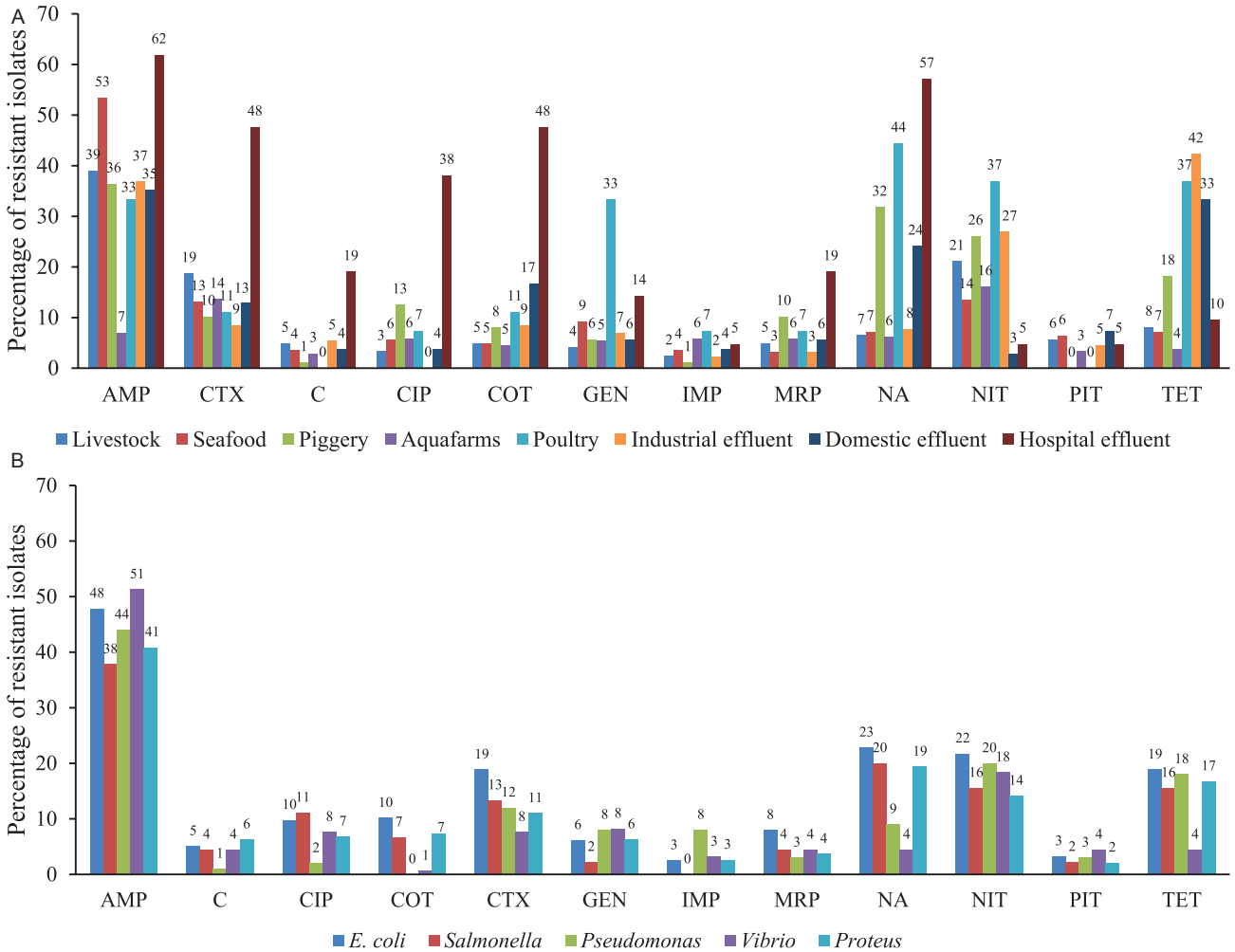

Figure. Resistance profile of Gram-negative bacteria: (**A**) Resistance pattern observed in different sources; (**B**) Resistance pattern observed in different genera. AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; COT, co-trimoxazole; CTX, cefotaxime; GEN, gentamicin; IMP, imipenem; MRP, meropenem; NA, nalidixic; NIT, nitrofurantoin; PIT, piperacillin/tazobactam; TET, tetracycline.

selected ARDs was performed in a thermocycler (Applied Biosystems, USA).

The presence of ARDs in the plasmid and prophage DNA from Gram-negative isolates whose genotype confirmed the presence of resistance genes was tested. Plasmid extraction was performed by the alkaline lysis method[21]. Prophage induction was performed using mitomycin C at a final concentration of 1 µg/ml (Sigma Aldrich, India). Phage DNA was extracted from the lysate obtained after prophage induction[22]. PCR-based detected for ARDs in these fractions was performed.

In this study, 1122 Gram-negative bacteria were isolated from 242 samples and the antimicrobial susceptibility patterns were studied (Figs. A and B). 74.6 per cent of the isolates (837 of 1122) were resistant to at least one of the antibiotics tested. Of the 1122 isolates, 498 were resistant to AMP (44.4%), 220 to NIT (19.6%), 156 to TET (13.9%), 158 to CTX (13.2%), 120 to NA (10.7%), 73 to GEN (6.5%), 66 to co-trimethoxazole (5.9%), 59 to CIP (5.3%), 43 each (3.8%) to chloramphenicol and IMP, 58 to meropenem (5.2%) and 48 to piperacillin/tazobactam (4.3%). A total of 342 isolates were resistant to a single antibiotic tested while 243 of the resistant isolates (21%) were concomitantly resistant to more than one class of antimicrobials with resistance to AMP with NIT encountered more frequently (26 isolates) followed by resistance to TET with NIT (17 isolates) and AMP and NA (14 isolates). Resistance to more than three groups of antimicrobials (multidrug resistance) was observed in 43 isolates (~4%) with resistance to β-lactams, TET, quinolones and NIT most often encountered (12 isolates). The resistance patterns in multidrug-resistant isolates are depicted in Table. Chloramphenicol has been suggested as a drug of choice against multi-drug resistant Gram-negative bacteria[23]. However, in our study, of the 43 isolates resistant to chloramphenicol, 41 were concomitantly resistant to other groups of antimicrobials including quinolones and cephalosporin.

AMR determinants for genes frequently reported to be mobile through elements such as plasmids and phages were detected in a small fraction (72 isolates) of the resistant isolates. Nearly 56 isolates harboured genes coding for β-lactamases and the determinants bla_{TEM} and bla_{CTX-M} were concomitantly detected in 10 of these isolates; 27 isolates harboured genes coding for TET resistance and multiple variants of the *tet*

Table. Resistance patterns observed in multidrug-resistant isolates

Number of isolates	AMP and/CTX	C	NA and/CIP	TET	GEN	COT	NIT
12	R		R	R			R
3	R	R	R				R
3	R		R		R		R
1	R	R			R		R
4	R			R	R		R
2	R		R	R		R	
1			R	R		R	R
2	R	R		R			R
1	R		R		R		R
2	R	R	R				R
2	R	R	R			R	
1	R	R	R		R		
1	R	R	R	R			
1	R		R		R	R	
1	R	R	R			R	R
2	R	R	R	R			R
3	R	R	R	R		R	R
1	R	R	R	R	R	R	R

AMP, ampicillin; CTX, cefotaxime; C, chloramphenicol; NA, nalidixic; CIP, ciprofloxacin; TET, tetracycline; GEN, gentamicin; COT, co-trimoxazole; NIT, nitrofurantoin

gene in the same culture were observed in 11 isolates. Sulphonamide resistance encoded by the *sul* genes was observed in 20 isolates with multiple variants of the *sul* genes observed in eight isolates. Determinants for fluoroquinolone resistance, qnrA, qnrB, qnrS and qepA were present in one, three, one and two isolates, respectively. Extended spectrum β-lactamase (ESBL) in combination with *sul* genes were detected in six isolates and ESBL with plasmid-mediated quinolone resistance (PMQR) genes in four. ESBL, *sul* and PMQR genes together were observed in four isolates while ESBLs with *tet* genes were detected in only one isolate.

The property of the ARD being plasmid encoded and/or inducible in the temperate phage fraction was studied. The bla_{CTX-M} genes were observed to be extremely mobile being present in the plasmid of 61 per cent (11 of 18) and packed in prophage fractions in 72 per cent (13 of 18) of the isolates. The bla_{TEM} variants were more mobile in phage fractions (69%) compared to being plasmid encoded (15%). Of the TET resistance determinants, *tet*A was plasmid encoded in about 80 per cent of the isolates and in the inducible phage fraction in 33 per cent of the isolates. The *sul* genes were completely chromosomal and were not present in either the plasmid or prophage.

Standards on recommended antimicrobial use for animals and training of personnel involved in farm practices are not readily available resulting in inappropriate and indiscriminate use of antimicrobials in this sector. Our study highlights the need to control resistance in human-associated environments by systematic surveillance and the institution of antimicrobial stewardship practices in these sectors.

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

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