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Resistance of *Escherichia coli* and Salmonella Isolated from Marine and Freshwater Fishes towards Carbapenems

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Abstract

The carbapenems are beta-lactam antimicrobial agents with an exceptionally broad spectrum of activity and are active against most *Enterobacteriaceae* including those that produce ampC β-lactamase. Resistance to carbapenems, especially carbapenems-resistant-*Enterobacteriaceae* (CRE) is a serious issue worldwide as it is extremely difficult to combat bacteria resistant to carbapenems. Fifty two isolates of *Escherichia coli* and twelve isolates of Salmonella (*invA* and *stn* gene positive) isolated from marine fish and freshwater fish were tested for their susceptibility to carbapenems namely meropenem, imipenem and ertapenem. A low percentage (3.9%) of *E.coli* showed resistance to meropenem, while 11.5 & 17% of *E.coli* and 8.3 & 25% of Salmonella showed intermediate sensitivity to meropenem and ertapenem, respectively. However, 100% of *E. coli* and Salmonella were sensitive to imipenem. Importantly, meropenem resistant *E.coli* was also found to be resistant to other antibiotics such as ciprofloxacin, gentamicin, nalidixic acid, norfloxacin and tetracycline. The results indicate that carbapenem resistance in *E. coli* and Salmonella isolated from fish was marginal but nevertheless needs continuous monitoring.

Keywords: Carbapenems, meropenem, ertapenem, imipenem, *Escherichia coli*, Salmonella, antibiotic resistance

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Introduction

Carbapenems were first introduced in 1980 and are frequently used as the last choice in treating serious infections caused by multidrug-resistant strains of Gram negative bacilli in intensive care units and in high risk wards (Zhanel et al., 2007). Emergence and dissemination of carbapenem resistance among *Enterobacteriaceae* represent a serious threat to public health worldwide (Kumarasamy et al., 2010; CDC, 2012; Motoseje, et al., 2012). New recommendations of Clinical Laboratory Standards Institute (CLSI, 2012) lowered the interpretative criteria (breakpoints) for determining susceptibility to meropenem, ertapenem and imipenem among *Enterobacteriaceae* (Table 1). In the context of the lowered carbapenems breakpoints, a study was taken up to determine the incidence of carbapenems resistance in *Escherichia coli*, a commonly encountered faecal indicator bacteria and Salmonella, a food borne pathogen, isolated from marine and freshwater fish. *E. coli* and Salmonella are members of *Enterobacteriaceae* and have been frequently reported from tropical freshwater and marine finfish (Nambiar & Surendran, 2003; Padmaja et al., 2005; Rao et al., 2006). Faecal pollution of fish either during harvest or post-harvesting handling is the prime source of *E. coli* in marine and freshwater fish. Resistance to carbapenems has been reported in *E. coli* (Tortola et al., 2005; Rogers et al., 2011) and Salmonella (Miriagou et al., 2003) and is often plasmid mediated. Genetic material conferring antibiotic resistance is readily transmitted between the members of *Enterobacteriaceae*. Mizan et al. (2002) showed that R-plasmids were transferred at a slow rate from Salmonella to *E.coli* but at a higher frequency from *E. coli* and *E. coli* $O_{157}H_7$.

Materials and Methods

Fifty two isolates of *E. coli* and twelve isolates of Salmonella were isolated from freshwater fish (n=11) and marine fish (n=12) procured from local fish markets in Visakhapatnam, Andhra Pradesh, India. Fish samples were analysed for *E. coli* as per

Feng et al. (2002). Marine and freshwater fish samples (25 g) were homogenized with Butterfield's phosphate-buffered water (225 ml) in a Stomacher blender and analysed employing the 3 tube MPN method using lauryl sulfate tryptose (LST) broth, brilliant green lactose bile broth (BGLB) and EC broth. The *E. coli* isolates were confirmed by streaking on Levine's eosin-methylene blue (L-EMB) agar and by performing IMVC tests. Salmonella were isolated as per Andrews et al. (2011) by homogenizing fish sample (25 g) in lactose broth (225 ml), followed by selective enrichment in Tetrathionate (TT) broth and Rappaport-Vassiliadis (RV) medium; selective plating on Bismuth sulfite (BS) agar, Hektoen enteric (HE) agar and Xylose lysine desoxycholate (XLD) agar and finally confirmed by performing biochemical tests.

PCR method that selectively amplifies a DNA fragment within the *invA, and stn* gene of Salmonella was used. The sequence of PCR primers was mentioned in Table 2. One ml of Salmonella culture (24 h at 37°C) grown in Brain Heart Infusion (BHI) broth was centrifuged (10,000 rpm, 10 min at 4°C). The cell pellet was resuspended in 200 µl of Tris-EDTA (TE) buffer, placed in a dry bath for 5 min at 95°C and the crude lysate was used as template DNA. The PCR amplification reactions were performed in a final volume of 25 µl. Each reaction mixture contained 0.4 pmol μl^{-1} concentration of primer, 200 mmol l^{-1} of dNTP (dATP, dCTP, dGTP and dTTP), 1X reaction buffer (20 mmol l^{-1} Tris-HCl (pH 8.0), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$), 1U of Taq polymerase and 5 µl of sample DNA. DNA amplification was carried out in Thermal Cycler (Minicycler, MJ Research, MA, USA) with the following reaction conditions; initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30s, 64°C for 30s, and 72°C for 30s for *invA* gene and 25 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for *stn* gene. A final extension of 5 min at 72°C was employed in both cases. Agarose gel analysis (Sambrook & Russell, 2001) of the amplified products of *invA* and *stn* genes was carried out by loading 10 µl of the PCR product in a 2% agarose gel containing 1 µg ml^{-1} ethidium bromide in 1X TAE buffer and electrophoresed. After appropriate migration with constant voltage of 5–10 V/cm, the agarose gel was scanned using a gel documentation system (GelDoc, Biorad)

Antimicrobial susceptibility of all the isolates was performed on Mueller Hinton (MH) agar plates by standard Kirby Bauer disk diffusion method (Bauer et al., 1966) using meropenem (10 µg), imipenem (10 µg), and ertapenem (10 µg) discs (HiMedia). *E. coli* culture was inoculated to tryptone broth and incubated at 37°C for 4-6 h until moderate turbidity

Table 1. Previous and Current Clinical and Laboratory Standards Institute (CLSI) interpretative criteria (breakpoints) for carbapenem susceptibility and *Enterobacteriaceae* (CSLI, 2012)

Carbapenem	Previous breakpoints MIC (µg ml^{-1})*		Current breakpoints MIC (µg ml^{-1})**	
	Susceptible	Resistant	Susceptible	Resistant
Meropenem	≤ 4	≥ 16	≤ 1	≥ 4
Ertapenem	≤ 2	≥ 8	≤ 0.25	≥ 1
Imipenem	≤ 4	≥ 16	≤ 1	≥ 4

* M 100-S19; ** M 100- S22

Table 2. Primer sequences and reaction parameters

Primer sequence	Target	Amplicon Size (bp)	Annealing temperature (°C)	References
GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	*invA*	284	64	Rahn et al., 1992
CTTTGGTCGTAAAATAAGGCG TGCCCAAAGCAGAGAGATTC	*stn*	260	55	Makino et al., 1999

Table 3. MIC levels of meropenem to *Escherichia coli* isolated from freshwater and marine fish

	E.coli isolates from Marine fish	*E.coli* isolates from Freshwater fish
Sensitive	0.125 to 0.75 μg ml^{-1}(0.39±0.19)*	0.125 to 0.75 μg ml^{-1} (0.37±0.2)
Intermediate	1.5 - 2 μg ml^{-1} (1.6±0.22)	1.5 μg ml^{-1}
Resistant	8 μg ml^{-1}	6 μg ml^{-1}

*Mean± SD

developed. 100 μl of the *E. coli* culture was spread plated on MH Agar and the inoculums was allowed to dry for 10-15 minutes. Meropenem, imipenem and ertapenem discs was aseptically deposited on the MH agar plates and incubated at 37°C for 16-18 h. The zones showing inhibition were measured and interpreted based on CLSI interpretive criteria:- Meropenem & Imipenem: sensitive > 23 mm, resistant < 19 mm; Ertapenem: sensitive > 22 mm, resistant < 18 mm (CLSI, 2012). The same procedure was followed for Salmonella isolates. The minimum inhibitory concentration (MIC) of meropenem was determined using Ezy MIC strip 0.002 μg ml^{-1} to 32 μg ml^{-1} (HiMedia). The MIC of meropenem was read at the point (concentration) of complete inhibition of *E. coli* growth. Meropenem resistant *E. coli* isolates were additional tested for antimicrobial susceptibility to Ciprofloxacin (5 μg), Chloramphenicol (30 μg), Gentamicin (10 μg), Nalidixic acid (30 μg), Norfloxacin (10 μg), and Tetracycline (30 μg) discs.

Results and Discussion

E. coli was detected in all the marine fish and freshwater fish samples. Fifty two isolates of *E. coli* were obtained from marine fish (n=29) and freshwater fish (n=23). Response of *E. coli* (n=52) to the carbapenems *viz.,* meropenem, ertapenem and imipenem was tested and the result is depicted in Fig. 1. All the *E.coli* isolates were sensitive to imipenem. Sensitivity to meropenem and ertapenem was 84.6 and 82.7%, respectively. Resistance of *E.coli* isolates was observed only towards meropenem, albeit at a low level of 3.9%. Intermediate level of sensitivity was 11.5% to meropenem and 17.3% to ertapenem. The results indicate that the resistance to carbapenems in *E.coli* isolated from freshwater and marine fish was at a low level and was only towards meropenem. Pfaller & Jones (1997) reported that 99 to 100% of isolates of Gram negative bacteria were susceptible to meropenem. Meropenem was found to be the most active of the ten antimicrobial agents tested against a total of 212 Gram-negative isolates of which 125 were confirmed by reference methods to be extended spectrum β-lactamase (ESBL) producers (Jones et al., 2002). Moreover, meropenem has good CSF penetrability and useful in treatment of childhood meningitis and infections in neutropenic children (Shah & Narang, 2005).

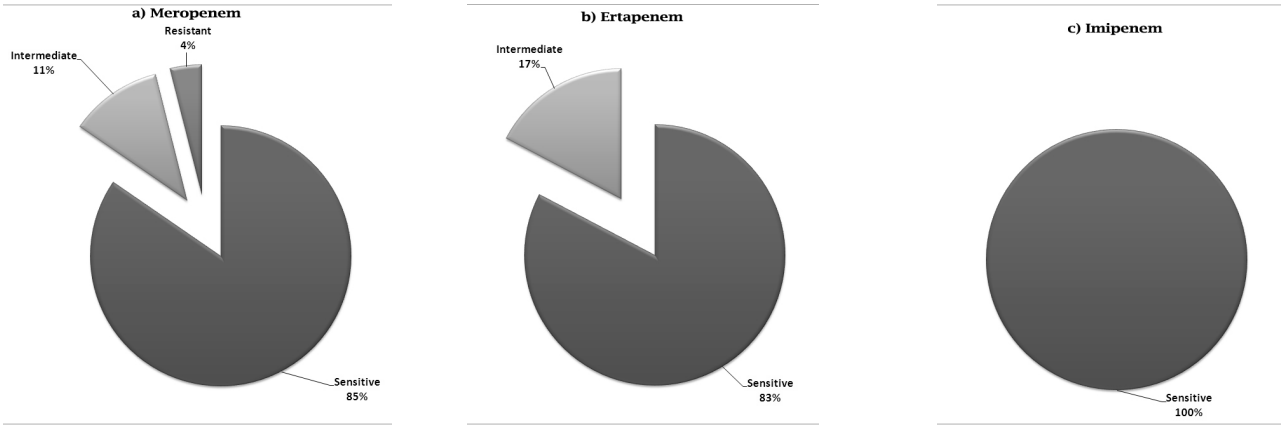

Fig. 1. Response of *E.coli* isolates (n=52) to carbapenems; a) Meropenem; b) Ertapenem c) Imipenem

Fish source wise, certain differences were observed in the *E. coli* isolated from freshwater and marine fish in their response to carbapenems. All the *E. coli* isolates from both marine (n=29) and freshwater fish (n=23) were sensitive to imipenem but differences in sensitivity pattern of *E. coli* isolates to meropenem and ertapenem were observed. Only 79.3% of *E. coli* isolates from marine fish were sensitive to meropenem whereas 91.3% of *E. coli* isolates from freshwater fish showed sensitivity to meropenem. Contrarily, 65.2% of *E. coli* isolates from freshwater fish were sensitive to ertapenem whereas a higher percentage of *E. coli* isolates (96.6%) from marine fish showed sensitivity to ertapenem. The reason for lower levels of sensitivity in *E. coli* isolated from marine fish towards meropenem and freshwater fish towards ertapenem is not known. Although the marine and freshwater fish were procured from the local markets, their origins were different; marine fish were sourced from the wild while the freshwater fish were sourced from aquaculture farms. The carbapenem resistance in *E. coli* might have been either due to co-acquired property via acquisition of plasmid mediated resistance for other antibiotics (cross resistance) or might have been transferred from clinical isolates. Resistance of *E.coli* isolated from fish and fishery environment to antibiotics other than carbapenems has been previously reported. A study on the antibiotic resistance in *E. coli* isolated from freshwater fish from Andhra Pradesh showed that 6, 24, 30 and 34 of the *E. coli* isolated were resistant to ciprofloxacin, nalidixic acid, tetracycline and nitrofurantoin, respectively but none of the isolates showed resistance to chloramphenicol, norfloxacin and gentamicin (Padmaja et al., 2005). The emergence of carbapenem resistant strains may have serious implications because of the limitation of therapeutic choices and by facilitation of the spread of resistant genes in the community.

The minimum inhibitory concentration (MIC) of meropenem was read at the point of complete inhibition of *E. coli* growth vis-à-vis meropenem concentration and the results presented in Table 3. The MIC for *E. coli* isolates sensitive to meropenem ranged between 0.125 to 0.75 μg ml^{-1}. The MIC of meropenem resistant strain isolated from marine fish was 8 μg ml^{-1} while that from freshwater fish was 6 μg ml^{-1}. It is pertinent to note that these *E. coli* isolates would have been classified as not resistant based on previous cut off value of ≥16 μg ml^{-1}. In the context of the new guidelines of CLSI (2012), the screening of environmental and clinical isolates of *E. coli* for carbapenems resistance assumes greater importance in order to ascertain the present status of their sensitivity vis-à-vis carbapenems.

Carbapenem resistant *Enterobacteriaceae* often carry genes that confer high levels of resistance to many other antimicrobials leaving very limited therapeutic options (CDC, 2012). Meropenem resistant *E. coli* isolated from marine fish was found to be resistant to ciprofloxacin, gentamicin, nalidixic acid, norfloxacin and tetracycline whereas the isolate from freshwater fish showed resistance to ciprofloxacin, norfloxacin, tetracycline but intermediate sensitivity to gentamicin and nitrofurantoin. However, meropenem resistant *E. coli* was found to be sensitive to chloramphenicol and as discussed earlier was also sensitive to imipenem.

All the Salmonella (n=12) isolated from fish were positive for the presence of *invA* genes and *stn* genes indicating that all the isolates were virulent Salmonella, thereby stressing the imminent danger (Fig. 2). The *invA* and *stn* genes of Salmonella are responsible for invasion of the gut epithelial tissue and enterotoxic effect to epithelial cells of humans. Rao & Surendran (2013) reported that majority of *V. cholerae* isolates from black tiger shrimp farms do not carry the *ctxAB* genes and are non-choleratoxigenic.

In the present study, none of the Salmonella isolates showed resistance to meropenem, imipenem and ertapenem. All the Salmonella isolates were sensitive to imipenem but 25 and 8.3% of Salmonella showed intermediate sensitivity to ertapenem and meropenem, respectively. MIC of meropenem ranged from 0.125 to 0.75 μg ml^{-1} with a mean value of

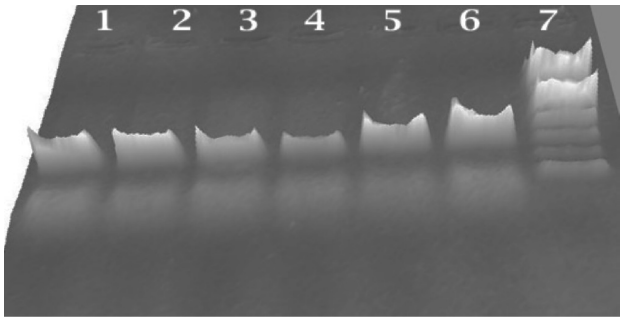

Fig. 2. Salmonella isolates showing the amplification of *invA* gene (284 bp amplicon)
Lane 1 to 5: Salmonella isolates from fish; Lane 6: Salmonella Type culture; Lane 7: 100 bp Marker

0.38±0.19 μg ml^{-1}. Human health is threatened by the emergence of salmonella resistant to antibiotics. Use of antibiotics in aquaculture favours the selection of resistant *Salmonella* strains (Zhao et al., 2003). Kumar et al. (2009) reported that Salmonella isolated from fish were found to be resistant to sulfamethizole, carbenicillin, oxytetracycline and nalidixic acid.

Amongst the carbapenems, only a low percentage of *E. coli* (3.9%) isolated from marine and freshwater fish showed resistance to meropenem. The *E. coli* and Salmonella isolates from fish showed resistance neither to ertapenem nor imipenem. The results indicate that carbapenem resistance in *E. coli* and Salmonella isolated from fish was marginal but nevertheless needs continuous monitoring. The elimination of faecal contamination both during harvest and post-harvest handling should be the prime strategy for minimizing the incidence of *E. coli* and Salmonella in marine and freshwater fish.

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References

Andrews, W. H., Jacobson, A. and Hammack, T. (2011) Salmonella. In: Bacteriological Analytical Manual (BAM), U.S. Food and Drug Administration, http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm (Accessed 12 Feb 2013)

Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M. (1966) Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Path. 45: 493-496

CDC (2012) 2012 CRE Toolkit – Guidance for Control of Carbapenem resistant Enterobacteriaceae (CRE), Centers for Disease Control and Prevention, Atlanta, GA, USA, pp 1-29, www.cdc.gov/hai/organisms/cre/cre-toolkit/ (Accessed 31 July 2013)

CLSI (2012) Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty Second Informational Supplement (January 2012). CLSI document M100-S22. Wayne, Pennsylvania, 2012. Vol. 32, No.3

Feng, P., Weagant, S. D., Grant, M. A., Burkhardt, W. (2002) Enumeration of *Escherichia coli* and the Coliform Bacteria. In: Bacteriological Analytical Manual (BAM), U.S. Food and Drug Administration, http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm (Accessed 12 Feb 2013)

Jones, R. N., Rhomberg, P. R., Varnam, D. J. and Mathai, D. (2002) A comparison of the antimicrobial activity of meropenem and selected broad-spectrum antimicrobials tested against multi-drug resistant Gram-negative bacilli including bacteraemic Salmonella spp.: initial studies for the MYSTIC program in India. Int. J. Antimicrob. Agents. 20: 426-431

Kumar, R., Surendran, P. K. and Thampuran, N. (2009) Analysis of antimicrobial resistance and plasmid profiles in *Salmonella* serovars associated with tropical seafood of India. Foodborne Pathog. Dis. 6: 621-625

Kumarasamy, K. K., Toleman, M. A., Walsh T.R. et al. (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: A molecular, biological, and epidemiological study. Lancet Infect. Dis. 10: 597–602

Mizan, S., Lee, M. D., Harmon, B. G., Tkalcic, S. and Maurrer, J. J. (2002) Acquisition of antibiotic resistance plasmids by Enterohaemorhhagic *E.coli* $O_{157}H_7$ within rumen fluid. J. Food Protect. 65: 1038-1040

Miriagou, V., Tzouvelekis, L. S., Rossiter, S., Tzelepi, E., Angulo, F. J., and Whichard, J. M. (2003) Imipenem resistance in a *Salmonella* clinical strain due to plasmid-mediated Class A Carbapenemase KPC-2. Antimicrob. Agents Chemother. 47: 1297–1300

Motoseje, L.F., Bryce, E., Roscoe, D., Boyd, D.A., Embree, J., Gravel, D., Katz, K., Kibsey, P., Kuhn, M., Mounchili, A., Simor, A., Taylor, G., Thomas, E., Turgeon, N., Mulvey, M.R (2012) Carbapenem-resistant Gram-negative bacilli in Canada 2009-10: results from the Canadian nosocomial infection surveillance program. J. Antimicrob. Chemother. 67: 1359-1367

Nambiar, V. N. and Surendran, P. K. (2003) Microbial hazards in fish sold in the retail markets of Cochin. In: Seafood Safety (Surendran, P. K., Mathew, P. T., Thampuran, N., Nambiar, V. N., Joseph, J., Boopendranath, M.R., Lakshmanan, P.T., Nair, P.G.V., Eds.) pp 491-496, Society of Fisheries Technologists (India), Cochin

Padmaja, G., Rao, B. M. and Surendran, P. K. (2005) Microbial risk assessment of freshwater fish for internal trade: a preliminary study. In: Sustainable Fisheries Development Focus on Andhra Pradesh (Boopendranath, M. R., Mathew, P. T., Gupta, S. S., Pravin, P. and Jeeva, J.C. Eds), pp 208-214, Society of Fisheries Technologists (India), Cochin

Pfaller, M. A. and Jones, R. N. (1997) A review of the *in vitro* activity of meropenem and comparative antimicrobial agents tested against 30,254 aerobic and

anaerobic pathogens isolated worldwide. Diagn. Microbiol. Infect. Dis. 28: 157-163

Rao, B. M., Gupta, S. and Surendran, P. K. (2006) Microbial quality of post-tsunami seafood of the Visakhapatnam coast. Fish. Technol. 43: 192–197

Rao, B. M. and Surendran, P. K. (2013) Detection of ctx gene positive non-O1/non-O139 *V. cholerae* in shrimp aquaculture environments. J. Food Sci. Technol. 50: 496-504

Rogers, B. A., Sidjabat, H. E. and Paterson, D. L. (2011) Escherichia coli O25b-ST131: a pandemic, multiresistant, community-associated strain. J. Antimicrob. Chemother. 66: 1-14

Sambrook, J., and Russell, W. D. (2001) *Molecular cloning: a laboratory manual*. pp 5.1–5.14 Cold Spring Harbor Laboratory Press, New York, USA.

Shah, D. and Narang, M. (2005) Meropenem. Ind. Pediatr. 42: 443-450

Tortola, M.T., Lavilla, S., Miro, E., Gonzalez, J.J., Larrosa, N., Sabate, M., Navarro,F. and Prats, G. (2005) First detection of a carbapenem-hydrolyzing metalloenzyme in two *Enterobacteriaceae* isolates in Spain. Antimicrob. Agents Chemother. 49: 3492-3494

Zhanel, G. G., Wiebe, Ryan, W., Dilay, Leanne, D., Thomson, Kristjan, T., Ethan, R., Hoban, D.J., Noreddin, A. M. and Karlowsky, J. A. (2007) Comparative Review of the Carbapenems. Drugs, 67: 1027-1052

Zhao, S., Datta, A. R., Ayers, S., Friedman, S., Walker, R. D. and White, D. G. (2003) Antimicrobial-resistant Salmonella serovars isolated from imported foods. Int. J. Food Microbiol. 84: 87–92