Global Journal of Pharmacology 8 (1): 73-79, 2014
ISSN 1992-0075

DOI: 10.5829/idosi.gjp.2014.8.1.8270

Resistance Patterns and Prevalence of the Aminoglycoside Modifying Enzymes in Clinical Isolates of Gram Negative Pathogens

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Abstract: In this nationwide study we investigated the occurrence of aminoglycoside resistance patterns and prevalence of the aminoglycoside modifying enzymes (AMEs), *aac(6)*, *ant(2)* and *aph(3)*, in clinical isolates of *Acinetobacter* species, *E. coli*, *Klebsiella* species and *Pseudomonas* species isolates from various clinical specimens. A total of 319 clinical specimens recovered from urine, blood, wound, catheter tips and sputum were collected and were processed for identification of bacterial isolates in these specimens. The selected bacterial isolates were examined for susceptibility to Potentox, cefepime, amikacin, tobramycin, meropenem, gentamicin, piperacillin plus tazobactam by disc diffusion method. AMEs were detected by polymerase chain reaction (PCR). A total of 255 Gram negative clinical isolates were recovered from clinical specimens that include 9.0 % of *Acinetobacter* species, 34.9% of *Escherichia coli,* 29.0% of *Pseudomonas* species and 27.0% of *Klebsiella* species. Among the 255 clinical isolates, 75.7% isolates were found to carry AMEs. The AMEs genes found were *aac(6)* (43.5 to 51.7%), *ant(2)* (17.4 to 20.2%) and *aph(3)* (5.6 to 10.1%) of the isolates. The most prevalent AMEs was *aac(6)*. Our data displayed that Potentox was the most active antibacterial agent against AMEs followed by meropenem. Potentox exhibited more than 93% susceptibility to all 3 types of AMEs (aac, ant & aph) whereas meropenem response was almost 20% lesser with susceptibility ranging not more than 73.4%. Piperacillin plus tazobactam was found to be the least active with less than 20% susceptibility. The susceptibility of other antibacterial agents varied between 20% to <40% %. In conclusion 75.7 % isolates carried AMEs that included *aac(6), ant(2) and aph(3)* which are responsible for resistance. Among the tested drugs, traditionally used aminoglycoside showed the maximum resistance. Surprizingly, broad spectrum antibiotics like meropenem, cefepime and piperacillin tazobactam also exhibited resistance to aminoglycoside modifying enzyme producing strains. However, in this study, Potentox showed excellent *in vitro* antibacterial activity up to 95 % of all isolates. We suggest that that Potentox which has been introduced recently into clinical settings would allow clinicinas to overcome the aminoglycoside resistance acquired by some bacterial strains.

Key words: Aminoglycoside Modifying Enzymes · Potentox · Susceptibility · Resistance

INTRODUCTION

Aminoglycosides represent highly potent, broad spectrum antibiotics that have been used for the treatment of life threatening Gram negative bacterial infections. They exert their antibacterial activity by inhibiting protein synthesis via binding to the 16S rRNA and by disrupting the bacterial cell membrane integrity [1]. However, over the past few years, the emergence of resistant strains has reduced the potential of aminoglycosides in empiric therapies [2]. A number of mechanisms of aminoglycosides resistance in Enterobacteriacae and non-fermenters have been known that includes reduced uptake or decreased cell permeability [3], alteration of the ribosomal binding site by rRNA methylases [4-6], overexpression of efflux pump [7-8] and production of aminoglycoside-modifying enzymes (AMEs) [9-11]. Among these mechanisms, aminoglycoside resistance is primarily mediated by AMEs that modify the drugs as a result poor binding to the ribosome and fail to trigger energy-dependent phase II allowing the bacteria to survive in the presence of the drugs [12]. There are three

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families of AMEs such as aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs) and aminoglycoside phosphotransferases (APHs) and expression of these enzymes results in high level resistance to all commonly used aminoglycosides namely gentamicin, amikacin, tobramycin, neomycin, kanamycin, netilmicin [5, 13].

Aminoglycoside resistance is increasing years after years, with a high alarming rate. There have been a number of reports on resistance to aminoglycosides in Pseudomonas species, *Escherichia coli, Klebsiella* species, *Acinetobacter* species [14, 15]. In Gram negative organisms, resistance to aminoglycosides such as amikacin, tobramycin and gentamycin was reported to vary from 32.6% to 83.6% which is mediated by AAC(6) and APH(2) activity [15-17]. Furthermore, resistance to tobramycin and amikacin is also mediated by an ANT(4) enzyme encoded by *ant(4* gene [18]. An earlier study from India, reported the prevalence of AAC(6')-I and AAC(3)-II was 42.8 % and 20.4 %, respectively [15]. However, surveillance study on aminoglycoside resistance due to AMEs are scanty.

In view of increasing incidence of aminoglycoside resistance and failure of monotherapy, a combination therapy may be the only notable therapeutic approach to treat infections caused by aminoglycoside resistant organisms [19]. The combination of aminoglycosides with β-lactams have been documented to be synergistic [20]. In this nationwide study we investigated the occurrence of aminoglycoside resistance patterns and prevalence of the AMEs, *aac(6)*, *ant(2)* and *aph(3)*, in clinical isolates of *Acinetobacter* species, *E. coli*, *Klebsiella* species and *Pseudomonas* species isolates from various clinical specimens and evaluated the response of various broad spectrum antibiotics on these strains.

MATERIALS AND METHODS

Clinical Isolates Collection: A total of 319 clinical specimens from various hospitals of India located in Utter Pradesh, Madhya Pradesh, Kolkata, Banglore, Himanchal Pradesh and Delhi between June 2012 to December 2013. The specimens were isolated from urine (20.4%; 65/319), blood (17.9%;57/319), wounds (22.2%;71/319), catheter tipsa (16.3%;52/319), sputum (23.2%;74/319). Among these, 255 Gram negative bacterial isolates that include *Acinetobacter* species (23), *Escherichia coli* (89), *Pseudomonas* species (74) and *Klebsiella* species (69) were recovered and identified using standard microbiological procedures.

Antibacterial Agents: A novel antibiotic adjuvant entity (AAE) comprising cefepime hydrochloride and amikacin sulphate herein after referred to as Potentox (Venus Remedies Limited, Baddi, India), cefepime (Rocephion, Hoffmann-Laroche Pharmaceutiical Limited, Basel, Switzerland), tobramycin (Tobraneg; Venus Remedies Limited, Baddi, India), amikacin (Alfakim; Ranbaxy Laboratries Limited, India), meropenem (Meronem, Astrazeneca Pharma India Ltd., Banglore, India) gentamicin (Ranbiotic Ranbaxy Laboratories, Gurgaon India), piperacillin plus tazobactam (Zosyn; Wyeth Pharmaceuticals, Mumbai, India) were included in the study. All dry powder drugs were reconstituted according to instructions of manufacturer. Working solutions were prepared using Mueller-Hinton broth (MH, Hi-Media, Mumbai, India).

Aminoglycoside Susceptibility Testing: Antimicrobial susceptibility test was carried out using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [21]. The disc for Potentox (37.5 µg), cefepime (30 µg), amikacin (30 µg), tobramycin (10 µg), meropenem (10 µg), gentamicin (10 µg), piperacillin plus tazobactam (110 µg) were obtained from Hi-Media (Mumbai, India). *P. aeruginosa* (ATCC 27853) served as a control for the disk diffusion.

DNA Isolation: DNA from all isolates was extracted as described previously [22]. Five ml of each at concentration of 10^{10} colony forming unit (cfu)/ml was used for the DNA isolation. DNA purity and concentrations were measured with spectrophotometer (260/280).

PCR for Genes Encoding AMEs: DNA of each isolate was exposed to polymerase chain reaction (PCR) to screen all 255 isolates for the presence of the aminoglycoside modifying genes, aac(6), ant(2) and aph(3), using the primers listed in Table 1. PCR amplification was performed using 200 pg of template DNA, 0.5 mM of dNTPs, 1.25 µM of each primer and 1.5 U of Taq polymerase (Banglore Genei) in a total volume of 25 µL. PCR amplification was done using Eppendorf thermocycler (Germany). Thereafter, 5 µl of each PCR product was analyzed on 1 % (w/v) agarose gel supplemented with ethidium bromide. The amplicons were then visualized on a UV transilluminator and photographed (BioRad, USA).

Table 1: Various primers used for amplification of aminoglycoside resistant genes from clinical isolates

Primer name	Primer sequence (5 to 3)	Target gene (s)	Bp	Reference
Aac (6)	5'-CAGGAATTTATCGAAAATGGTAGAAAAG-3'			
	5'-CACAATCGACTAAAGAGTACCAATC-3'	*Aac (6)*	369	9
Aph (3)	5'-GGCTAAAATGAG AATATCACCGG-3'			
	5'-CTTTAAAAAAT CATACAGCTCGCG-3'	*aph(3)*	523	25
Ant (2)	5'-AAGCACGATGATATTGATCTG-3'			
	5'-GGCATAGTAAAAGTAATCCCA-3'	*Ant (2)*	288	15

Table 2: Prevalence of the microorganisms in different clinical specimens

		Number of clinical specimens (319)				
Name of species	Total number of isolates	Urine(65)	Blood (57)	Wound (71)	Catheter tips (52)	Sputum (74)
Acinetobacter species	23	-	7	3	5	8
Escherichia coli	89	29	15	17	13	15
Pseudomonas species	74	4	6	25	8	31
Klebsiella species	69	23	13	20	5	8
Total	255	56	41	65	31	62

Table 3:The percentage of aminoglycosides resistance gene detected in clinical isolates isolates from various clinical specimens.

Clinical	*Acinetobacter* species (23)				*E. coli* (89)			*Pseudomonas* species (74)			*Klebsiella* species (69)	
specimens	*aac*(6)	*ant* (2)	*aph* (3)	*aac*(6)	*ant* (2)	*Aph* (3)	*aac*(6)	*ant*(2)	*Aph* (3)	*aac*(6)	*ant* (2)	*Aph* (3)
Urine	-	-	-	17	4	1	6	3	-	8	4	2
Blood	4	1	-	8	3	1	2	1	1	5	2	-
Wound	2	-	-	8	3	1	13	5	2	10	3	2
Catheter tips	2	1	1	7	3	1	3	2	1	2	1	2
Sputum	3	2	1	6	5	1	12	3	3	5	3	1
Gene detection in isolates No. (%)	11 (47.8)	4 (17.4)	2 (8.7)	46 (51.7)	18 (20.2)	5 (5.6)	36 (48.6)	14 (18.9)	7 (9.4)	30 (43.5)	13 (18.8)	7 (10.1)

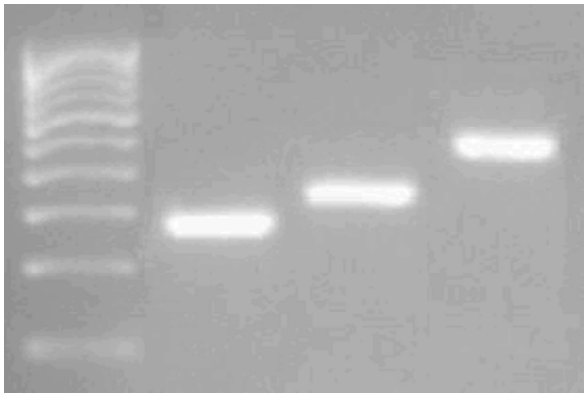

Fig. 1: Agarose gel showing PCR amplified products of aminoglycoside modifying enzymes.
Lane A, 100 bp DNA size marker; Lane B, *ant(2)* (288 bp); Lane C, *aac(6)* (369 bp); Lane D, *aph(3)* (523 bp).

RESULTS

As evident in Table 2, after processing of all 319 clinical specimens recovered from urine, blood, wound, catheter tips and sputum, 255 Gram negative clinical isolates were recovered that includes 23 isolates of *Acinetobacter* species (23/255; 9.0 %), 89 isolates of *Escherichia coli* (89/255; 34.9%) 74 isolates of *Pseudomonas* species (74/255; 29.0%) and 69 isolates of *Klebsiella* species (69/255; 27.0%). *E. coli* and *Klebsiella* spp. was most prevalent in urine whereas *Acinetobacter* spp. and *Pseudomonas* spp. were more prevalent in sputum.

Distribution of AMEs among Clinical Isolates: Out of 255 clinical isolates, 75.7% (193/255) isolates were found to express aminoglycoside modifying enzymes producing genes. As shown in Table 3, of 193 Gram neagtive isolates, 123/193 (63.7%) were acetyltransferase (AAC) followed by 49/193 (25.3%) adenyltransferase (Ant) and 21/193 (10.8%) phosphotransferase (Aph). Overall, the prevalence of *aac(6)* was 43.5 to 51.7% with maximum preveleance in *E.coli* , that of ant(2) was 17.4 to 20.2% with maximum prevalence in *E.coli* and aph (3) 5.6 to 10.1%, with maximum prevalence n *Klebsiella spp*. (Figure 1). The detailed distribution of aminoglycoside modifying genes in each clinical specimen is described in Table 3.

Table 4: Frequency of aminoglycosides resistance encoding genes detected in clinical isolates in relation with antibiotics sensitivity patterns

	aac (6) (123)			*ant* (2) (49)			*aph* (3) (21)		
Antibacterial agents	S	I	R	S	I	R	S	I	R
Potentox	94.3	1.6	4	93.8	4.1	2	95.2	4.8	-
Cefepime	32.5	17.8	49.6	32.6	24.5	42.8	33.3	19	47.6
Amikacin	38.2	13.8	47.9	38.7	20.4	40.8	38.1	15.8	47.6
Tobramycin	25.2	19.5	55.3	24.5	20.4	55.1	28.6	9.5	62
Gentamicin	23.6	16.3	60.1	26.5	22.4	51	23.8	19	52.4
Meropenem	72.3	9	18.7	73.4	10.2	16.3	71.4	4.7	23.8
Piperacillin plus tazobactam	17.1	13.8	69.1	22.4	18.4	59.2	19	28.6	52.4

Antibiotic Susceptibility Study: Table 4 shows the total aminoglycoside resistant encoding genes detected in these gram negative clinical isolates in relation with antibiotic susceptibility patterns. Our data displayed that Potentox was the most active antibacterial agent against AMEs followed by meropenem. Of a total of 123 *aac(6)* positive isolates, 94.3% isolates remained susceptible to Potentox whereas 72.3% isolates were susceptible to meropenem. Piperacillin plus tazobactam was found to be least active in aac(6) positive isolates with only 17.1% susceptible response. The susceptibility of other antibacterial agent varies from 23.6 % to 38.2 %.

The strains carrying *ant(2)* showed remarkable high level of susceptibility to Potentox (93.8%), meropenem (73.4%), amikacin (38.7%), cefepime (32.6%), gentamycin (26.5%), tobramycin (24.5%) and piperacillin plus tazobactam (22.4%) and showed high level of resistance against piperacillin plus tazobactam (59.2%), tobramycin (55.1%), gentamycin (51%), cefepime (42.8%), amikacin (40.8%), meropenem (16.3%) and potentox (2%). The *aph(3)* harboring isolates also exhibited maximal susceptibility against Potentox (95.2%) followed by meropenem (71.4%), amikacin (38.1%), cefepime (33.3%), tobramycin (28.6%), gentamycin (23.8%) and piperacillin plus tazobactam (19%) and demonstrated least resistance to meropenem (23.8%), amikacin (47.6%), cefepime (47.6%), each piperacillin plus tazobactam and gentamycin (52.4%) and tobramycin (62%).

DISCUSSION

In recent years, increasing occurrence of aminoglycoside resistant strains have imposed a major threat not only because of their ability to cause serious infections but also because of their increasing resistance to antimicrobial agents. The frequency of isolation of bacterial isolates from clinical specimens in different countries widely varies. In the present study only 4 species, *Acinetobacter* species (9.0 %), *Escherichia coli* (34.9%) *Pseudomonas* species (29.0%) and *Klebsiella* species (27.0%) were recovered from clinical specimens whereas other have isolated more [2].

Our data showed that the prevalence of AMEs in this study was 75.5%. PCR analysis of AMEs revealed the presence of three AMEs, *aac(6)*, *ant(2)* and *aph(3)* and all the isolates carry only single AME gene which is in agreement with other study conducted in Europe and USA which reported that the majority of isolates harboured only a single aminoglycoside modifying enzyme [23]. The aac(6) was most prevalent AMEs encountered in 63.7% of isolates which is similar to those of other reports from India and abroad [15, 24-25]. It is noteworthy that *aac(6)* enzyme has got notable attention as to be implicated in the resistance of kanamycins and tobramycin as well as amikacin and neitlmicin [26]. *Ant (2) was observed 25.3%* isolates which is in accordance with an earlier study from Iran in 250 isolates of *Pseudomonas aeruginosa* obtained from various clinical specimens which reported that *ant(2)* was prevalent detected in 28% of clinical isolates [27]. We found the least prevalence 10.8% of *aph(3)* which is similar to what has been observed by Vaziri *et al.* [27], who noted the *aph(3)* incidence in 11% of isolates. However, an earlier study from India in *Enterococcus* species, reported a high prevalence (40.4%) of *aph(3)* [28].

A number of earlier studies have noted the occurrence of aminoglycoside resistance in *Acinetobacter* species, *E. coli*, *Klebsiella* species and *Pseudomonas* species in various countries [14-15, 27]. Our data demonstrated resistance of AME producing strains to all amoniglycosides amikacin, tobramycin and gentamycin. Other studies conducted in Turkey and India have detected 49.7% and 55.1% resistance of Gram negative organisms to amikacin and 82.4% and 83.6 % resistance to tobramycin in India and Turkey, respectively [15, 29]. A study done by Estahbanati *et al*. [30] reported 53.3% of clinical isolates from Iranian burn patients were resistant to amikacin and 90.7% were resistant to gentamycin. The resistance of gentamycin varied from 94.5 % to 32.6

% [15, 29]. Surprisingly, we have observed an equal or higher resistance to other broad spectrum antibiotics like cefepime, piperacillin tazobactam and meropenem also. Our findings are in line with a study conducted in Japan by Morini *et al.* [25] who reported a high level of resistance to ceftazidime (76.9%) and piperacillin tazobactam (64.1%) .

In our study aminoglycoside resistance was high because most of the isolates harboured aminoglycoside modifying gene. Interestingly, in this study, 95 % of all isolates carrying AMEs showed susceptibility to Potentox. This may be due to synergism of aminoglycosides with β-lactams which enhanced the intracellular uptake of aminoglycosides by enhancing bacterial cell permeability. Furthermore, Potentox synergistically is assumed of having protein kinase inhibitor activity to inhibit the aminoglycoside modification through ATP-dependent O-phosphorylation, catalysed by aminoglycoside kinases particularly aminoglycoside phosphotransferases (Aphs). The enhanced susceptibility of Potentox is consistent with our previous studied where it has been demonstrated to have noticeable antibacterial activity [31, 32]. Its antibacterial activity has also been proved in animal model [33, 34].

CONCLUSION

In this study approximately 75.7 % isolates carried AMEs that included *aac(6), ant(2) and aph(3)* which are responsible for resistance with maximum prevalence of aac(6) type in *E.coli*. Among the tested drugs, traditionally used aminoglycoside showed the maximum resistance. However, in this study, Potentox showed excellent in vitro antibacterial activity upto 95 % of all isolates. We suggest that that Potentox which has been introduced recently into clinical settings would allow clinicians to overcome the aminoglycoside resistance acquired by some bacterial strains.

ACKNOWLEDGEMENT

Authors are thankful to institutes which provided clinical isolates.

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