AMINOGLYCOSIDE MODIFYING ENZYMES DETECTED IN STRAINS OF *ESCHERICHIA*, *KLEBSIELLA*, *PSEUDOMONAS* AND *ACINETOBACTER* IMPLICATED IN INVASIVE INFECTIONS IN NAIROBI, KENYA

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Abstract
Aminoglycoside resistance through the production of aminoglycoside modifying enzymes (AMEs) is common and thus of clinical importance. The presence of AMEs genes in gram-negative bacteria on plasmids, transposons and integrons facilitates the rapid acquisition of antibiotic resistance. This study aimed to characterize AMEs in antibiotic resistant strains of *Escherichia, Klebsiella, Pseudomonas* and *Acinetobacter* implicated in invasive infections in Nairobi, Kenya. The experimental design was a two point cross-sectional design comparing 54 clinical isolates obtained from the KEMRI laboratory collected in 2001 to 2006 and 54 clinical isolates from Aga Khan University Hospital collected in 2007 to 2008. The isolates were identified, tested for antimicrobial susceptibility to seven aminoglycosides then the AMEs were detected phenotypically and genotypically. The most prevalent AME gene detected was *acc(6)-Ib-cr* (45.9%) followed by *acc(3)-II* (25.9%, *aac(6)-I*(22.2%) and *aac(3)-I* (16.3%). Phenotypic studies showed that multidrug resistant *Pseudomonas aeruginosa* harboured numerous AMEs and 81% of the resistance was conferred by impermeability. Increase in aminoglycoside resistance by both naturally derived and semi synthetic antibiotics is alarming. Methods for monitoring their effectiveness should be instituted at the different healthcare system in Kenya.

Key words: Aminoglycoside, antibiotic resistance, aminoglycoside –modifying enzymes (AMEs)

1.0 Introduction

Aminoglycosides are a group of antibiotics that are characterized by the presence of an aminocyclitol ring linked to amino-sugars in their structure. They are particularly active against aerobic and facultative aerobic Gram-negative bacteria including members of family *Enterobacteriaceae*, *Acinetobacter, Pseudomonas* and *Enterobacter* species. They are most frequently used for treatment of invasive infections such as septicaemia, complicated intra-abdominal infections, complicated urinary tract infections, and nosocomial respiratory tract infections (Mingeot-Leclercq *et al.,* 1999). The resistance of clinical isolates to aminoglycoside antibiotics varies with the specific drug, the microorganism, its mechanism of resistance, the geographic area and many other factors (Vakulenko and Mobashery, 2003).

There are four mechanisms of aminoglycoside resistance; reduced uptake or decreased cell permeability, alteration of the ribosomal binding site by mutation expression of rRNA methylases and production of aminoglycoside-modifying enzymes (AMEs). It is noteworthy that more than one mechanism may be at play at the same given time in a bacterium in the case of some classes of drugs (Vakulenko and Mobashery, 2003; Shakil *et al.,* 2008). Production of AMEs is the most common mechanism of Aminoglycoside Resistance and thus of most clinical importance. The three families of enzymes: Aminoglycoside Acetyltransferases (AACs), Aminoglycoside Nucleotidyltransferases (ANTs) and Aminoglycoside Phosphotransferases (APTs) perform co-factor dependent drug modification in the bacterial cytoplasm. Modified aminoglycosides bind poorly to the ribosome and fail to trigger energy-dependent phase II allowing the bacteria to survive in the presence of the drug (Shaw *et al.,* 1993).

Aminoglycoside resistance genes are derived from bacterial genes which encode enzymes involved in normal cellular metabolism. There are over 50 different AMEs that have been identified and enzymatic modification results in high-level resistance. The level of resistance produced differs significantly in various microorganisms and individual strains and depends on many factors, including the amount of enzyme produced, its catalytic efficiency, and the type of aminoglycoside (Mingeot-Leclercq *et al.,* 1999; Vakulenko and Mobashery, 2003).

In Kenya, there is scarcityof data on aminoglycoside resistance in invasive infections although they form the mainstay drugs in our hospitals for treatment of serious nosocomial infections by *Acinetobacter* spp., *Pseudomonas aeruginosa*, and ESBL- producing *Enterobacteriaceae*. The aim of this study was to characterize Aminoglycoside resistant strains of *Escherichia*, *Klebsiella*, *Pseudomonas* and *Acinetobacter* implicated in invasive infections in Nairobi, Kenya.

2.0 Materials and Methods

The study was two point cross-sectional comparing old stored and new isolates. The stored isolates were obtained from Kenya Medical research Institute (KEMRI) laboratory and were collected during the period of 2001-2006 from two hospitals, Kenyatta National Hospital (KNH- old) and Aga Khan University Hospital (AKUH-old). New isolates were collected from Aga Khan University Hospital (AKUH-new) in 2007-2008. The sample size was determined using the Fisher *et al.* (1925) formula. A total sample size of 108 was determined where; 41 samples were *E. coli*, 35 *Klebsiella*, 21 *P. aeruginosa* and 11 *A. baumannii.* Specimen sources included; Intravenous blood, urine, sputum, tracheal aspirates, pus swabs, cerebral spinal fluid (CSF), catheters, and high vaginal swabs (HVS). Identification was done by the five tube method on triple sugar iron agar, sulphur indole motility agar, Simmon's citrate agar, MRVP broth and urea agar. Gram stain test were also done to confirm morphological characteristics.

Antibiotic susceptibility to seven aminoglycosides; amikacin (30µg), gentamicin (10µg), kanamycin (30µg), neomycin (30µg), streptomycin (10µg), tobramycin (10µg) from Oxoid Limited United Kingdom and High level Resistance (HLR) spectinomycin (300µg) Rosco Diagnostica, Denmark. The tests were carried out using the disc diffusion method on Mueller Hinton Agar and incubated at 37°C for 18-24 hours. Disk susceptibility tests were interpreted according to the guidelines provided by the manufacture's interpretation charts. *Escherichia coli* ATCC 25922 with known MICs was used as Standard control organism.

DNA Extraction was carried out whereby an 18-24hr single colony of each isolate was suspended in 1 ml of sterile distilled water, which was then heated at 95°C for 10 minutes. After heating, centrifugation was done at 14,000 rpm for 6 minutes at 4°C. The DNA-containing supernatant was extracted and used as the source of template for

further PCR amplification experiments.

Selected isolates were tested for AMEs by using the PCR primers listed in Table 1. PCR amplification reactions was performed in a volume of 25 µl containing 12 µl of Qiagen PCR Master Mix (Qiagen GmbH, Hilden, Germany), 1.0 µM concentrations of each primer, 6µl of PCR water and 5 µl of DNA template. PCR products were analysed by gel electrophoresis at 100V for 1½ hours in a 2% agarose gel stained in Ethidium bromide. Bioline Hyperladder 1 was used as the standard marker.

Ethical clearance was obtained from Kenya Medical Research Institute (KEMRI) Scientific Committee and Ethical Review Committee and Aga Khan University Hospital (AKUH) Scientific and Ethical Review Committees.

3.0 Results

Aminoglycoside susceptibility testing of *E. coli, Klebsiella* spp. and *Acinetobacter baumannii* showed susceptibility to amikacin and HLR spectinomycin and relative resistance to the kanamycin, tobramycin, streptomycin, gentamicin and neomycin. The isolates collected from urine, blood, pus swabs and tracheal aspirates specimens showed resistance to streptomycin, kanamycin, gentamicin and tobramycin and susceptibility to amikacin and HLR spectinomycin. Overall, the isolates from Aga Khan University Hospital (AKUH- New) collected in 2007-2008 showed resistance to streptomycin (87%), kanamycin 81%), gentamicin (69%), tobramycin (65%), neomycin (62%), amikacin(46%) and spectinomycin(36%) respectively. The AKUH- old isolates collected in 2001- 2006 showed resistance to neomycin (36%), kanamycin (34%), gentamicin (32%), streptomycin (30%), tobramycin (26%), spectinomycin (11%), and amikacin (4%). The KNH- old isolates showed resistance to neomycin (47%), streptomycin (36%), kanamycin (34%), gentamicin (32%), tobramycin (26%), spectinomycin (17%), and amikacin (2%), respectively (Figure 1).

There was a remarkable increase in resistance of seven Aminglycoside antibiotics tested on the four Gram negative bacteria from Aga Khan University Hospital isolates collected over eight years (2001-2008). Kanamycin showed 68% increase in resistance, amikacin showed 40% increase, streptomycin 57%, gentamicin 37%, tobramycin 39%, neomycin 26% and HLR spectinomycin 24% (Table 2).

Aminoglycoside Modifying Enzymes (AMEs) were detected phenotypically as described by Livermore *et al.* (2001). It is possible to determine the presence of AMEs by testing the susceptibility of isolates against a range of clinically available aminoglycosides as a pattern of resistance emerges which is unique to a specific enzyme. Based on this phenotypic interpretation *P. aeruginosa* had the most AME genes seen to be conferring 81% resistance by impermeability (Table 3). A total of six aminoglycoside modifying enzymes (AMEs) were detected based on there resistance to selected Aminoglycosides determining the number of isolates per primer. The results show that majority of the MDR *P. aeruginosa* contain 83% of the aminoglycoside modifying enzymes genes tested while *A. baumannii* isolates contained the 16% AME genes. Some of the isolates were found to contain more than one AME gene and are of great interest (Table 4).

PCR products for *AAC(6')-1b-cr* and *AAC(3)-IIa* genes showed they amplified at 509bp and 300bp (Plate 1 and 2) respectively. *AAC(6')-1b-cr* confers resistance to Aminoglycosides; kanamycin, tobramycin and amikacin, and Quinoloes; nalidixic acid, ciprofloxacin and norfloxacin. *AAC(3)-IIa* confers resistance to gentamicin and tobramycin. Isolates with these resistance profiles were selected from both stored KEMRI isolates collected in 2001-2006 and AKUH- new collected in 2007-2008. *AAC(6')-1b-cr* gene had the largest number of micro-organisms test positive (60%).

4.0 Discussion and Conclusion

In Kenya, aminoglycosides most widely used clinically are gentamicin, streptomycin and kanamycin which showed the greatest percentages of resistance. AKUH- New isolates showed the highest percentages of resistance with 87%, 81% and 69% resistance to streptomycin, kanamycin and gentamicin, AKUH- old isolates showed 30%, 34% and 32% and resistance to streptomycin, kanamycin and gentamicin and finally KNH-old isolates showed 36%, 34% and 32% resistance to streptomycin, kanamycin and gentamicin. A study by Över *et al.,* (2001) tested 696 Gram-negative bacteria for resistance to aminoglycosides and they found resistance rates to be very high for Gentamicin

(94.5%). In a similar study by Miró *et al.,* (2008) in Spain during a period of 3 months 803 *Enterobacteriaceae* isolates were tested against aminoglycosides and the isolates were most resistant to streptomycin 42.6%, followed by kanamycin 12.8% and gentamicin 8.4%. These high levels of resistance to streptomycin, gentamicin and kanamycin may be attributed to their prolonged and continuous use.

The present study confirms that there exists a link between the type of aminoglycoside used and the kind of aminoglycoside resistance mechanism that will prevail. Notable about the phenotypic characterisation was that MDR *P. aeruginosa* was seen to harbour numerous AME genes and confers 81% resistance by impermeability. In a study by Poole, (2005) the percentage incidence of *P. aeruginosa* most prevalent AMEs as follows Aac6')-II 18.39% and Ant(2")-I 11.87% while impermeability played the most part in conferring resistance with 26.15% incidence. The occurrence of numerous AME genes in MDR *P. aeruginosa* was of great interest this is because more than one mechanism of aminoglycosides resistance may be at play at the same given time. *P. aeruginosa* has a very active efflux system causing the reduction of intracellular concentration of aminoglycosides by the outer membrane changes in permeability and inner membrane decrease in transport leading to trapping the drug. The presence of AMEs in integrons and transposons may explain the multi-drug resistance of many aminoglycoside-resistant *P. aeruginosa* isolates (Poole, 2005).

The genotypic results of the six AMEs amplified by PCR showed the most widespread AME in the present study was *AAC(6')-Ib-cr* (45.9%), followed by *AAC(3)-II* (30.9%), *AAC(6')-II* (25.9%), *AAC(6')-I* (22.2%), and *AAC(3)-I* (16.3%). No Ant(4')-IIb enzyme was detected. A study on molecular characterisation of aminoglycosides resistance in Spain, (2008) on *Enterobacteriaceae*, *AAC-3-IIa* gene was 10% prevalent, *AAC-6'-Ib* was 3.8%, and *AAC-6'-Ic* was 0.3%. Twenty two percent of the strains presented more than one enzyme (Miró *et al.,* 2008). The enzyme Aac(6')-Ib-cr was the most widespread. In the micro-organisms tested in this study, the enzyme occurred at a frequency of 22% in *KlebsiellA* spp., 19% in *P. aeruginosa*, 14% in *E. coli* and 5% in *A. baumannii*. The discovery of the *cr* variant of the aminoglycoside-(6)-N-acetyltransferase (*AAC[6']-Ib-cr*) gene confers resistance against two unrelated classes of antibiotics-aminoglycoside and quinolone- by changing two amino acids Trp102Arg and Asp179- Tyr. The clinical appearance of *AAC(6')-Ib-cr*, including its molecular and phenotypic characteristics, and its association with other antibiotic resistance genes have not been clarified to date (So Youn Shin *et al.,* 2009) . Moreover, there has been no recent study on the prevalence of *AAC(6')-Ib-cr* in isolates from Kenya. The *AAC(6')-Ib-cr* gene has been found in a gene cassette located inside class 1 integron (Fihman *et al.,* 2008). This shows that the gene is capable of rapid horizontal transfer between *Enterobacteriaceae* isolates.

Aac(6')-I enzyme is also quite important as it has gained attention for conferring resistance to naturally occurring Kanamycins and Tobramycin as well as to their semi-synthetic derivatives Amikacin and Neitlmicin. A positive correlation between increased Amikacin use and the occurrence of enzyme-mediated resistance has been described. Amikacin a semi-synthetic aminoglycoside has an acylated N-1 group which makes it a poor substrate for a number of modifying enzymes (Schmitz *et al.,* 1999). The low incidence of Aac (6')-I in Italian and German hospitals reflects the low percentage of Amikacin usage (15.8% and less than 10% of the total aminoglycosides usage, respectively) (Neonakis *et al.,* 2003). Here in Kenya, hospitals are extensively using Amikacin in treatment of severe nosocomial infections thus increasing the level of resistance by 40% over the years as reported in this study. This may be attributed to the presence of this enzyme which is not only highly transferable as it is located within integrons and transposons but has been seen to co-exists very frequently with other antibiotic-inactivating enzymes such as ESBLs (Neonakis *et al.,* 2003).

The future of unravelling more mechanisms of Aminoglycoside resistance lies in the determination of the 3D atomic structure of AMEs by X-crystallography. Four AMEs crystal structures have been reported namely; Aac(3), Aac(6'), Ant(4') and Aph(3')-IIIa (Neonakis *et al.,* 2003). This information has been useful in the application of structural modifications of aminoglycosides resulting in reduction of the modified antibiotic to bind to the target RNA due to unfavourable steric and/or electrostatic interactions. Attempts to make semi-synthetic aminoglycosides that circumvent resistance enzymes have been done although these await further experimentation. Examples of these are Dibekacin which lacks the 3'-hydroxyl group and therefore does not substrate for Aph(3') compounds. Amikacin and Isepamycin have an acylated N-1 group which makes them poor substrates for several AMEs (Kotra *et al.,* 2000).

5.0 Conclusion

This study showed that the older naturally derived aminoglycoside antibiotics like streptomycin, kanamycin, and gentamicin showed very high levels of resistance in the clinical isolates of genera *Escherichia*, *Klebsiella, Pseudomonas* and *Acinetobacter*. MDR *Pseudomonas aeruginosa* was detected in the study and showed resistance to Carbapenems. The occurrence of numerous AME genes in MDR *P. aeruginosa* was of great interest indicating possibility of more than one mechanism of aminoglycosides resistance to be at play at the same given time. Presence of AMEs was established both phenotypically and genotypically. The genes detected were *AAC(6')-Ib-cr* (45.9%), followed by *AAC(3)-II* (30.9%), *AAC(6')-II* (25.9%), *AAC(6')-I* (22.2%), and *AAC(3)-I* (16.3%).

6.0 Acknowledgement

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Table 1: Selected Aminoglycoside resistance genes and Integrons sequences detected by PCR

AME genes (Aminoglycoside Resisted)	**F/ R Primer**	**PCR cycle**
aac(6')-I (Tob, Amk, Kan)	TATGAGTGGCTAAATCGAT/ CCCGCTTTCTCGTAGCA	94°C 15min, (94°C 45s, 55°C 45s, 72°C 45s) 34 cycles, 72°C 10min
aac(6')-II (Tob, Amk, Kan)	CGCTTGTTGATTTGCTGCT GTTCGC/TTGAAACGACCT TGACCTTCCG	94°C 15min, (94°C 45s, 55°C 30s, 72°C 1min) 30 cycles,72°C 10min
aac(6')-1b-cr (Tob, Amk, Kan+ NA, Cip, Nor)	TTGCGATGCTCTATGAGTG GCTA/ CTCGAATGCCTGG CGTGTTT	94°C 5min, (94°C 45s, 55°C 45s, 72°C 1min) 36 cycles, 72°C 10min
aac(3)-I (Gen, Tob)	AGCCCGCATGGATTTGA/ GGCATACGGGAAGAAGT	94°C 15min, (94°C 1min, 55°C 1min, 72°C 1min) 30 cycles, 72^{0}C 10min
aac(3)-IIa (Gen, Tob)	GCTAAACTCCGTTACC/ TAGCACTGAGCAAAGCC	94°C 15min, (94°C 45s, 60°C 30s 72°C 90s) 30 cycles, 72°C 10min
ant(4')-IIb (Tob, Kan)	GAGAACCCATATGCAACA TACTATCGCC/ TAGAATTCT AGCGCGCAC TTCGCTCTTC	94°C 15min, (94°C 1min, 58°C 30s, 72°C 1min) 35 cycles, 72°C 10min

Tob- Tobramycin, Kan- Kanamycin, Gen- Gentamicin, Amk- Amikacin, NA- Nalidixic acid, Cip- Ciprofloxacin, Nor- Norfloxacin

Table 2: Aminoglycoside resitance of Aga Khan University Hospital isolates collected 2001-2008

Isolate Source	Year of Collection	Percentage prevalence of resistance (%) AK	K	CN	S	TOB	NEO	SPCT
AKUH -new	2007-2008	4681	69	87	65	62	35	
AKUH- old	2001-2006	4	34	32	30	26	36	11
% Increase		**40**	**68**	**37**	**57**	**39**	**26**	**24**

AK-Amikacin, K-Kanamycin, CN-Gentamicin, S-Streptomycin, TOB-Tobramycin, NEO-Neomycin and SPCT-Spectinomycin.

Table 3: Phenotypic Characterisation of Aminoglycoside resistance profiles of E. coli, Klebsiella spp. and Pseudomonas aeruginosa

Test M.O	GEN	TOB	AMK	KAN	NEO	Gene present	Frequency
E. col	S	S	S	S	S	Classical*	-
	R	S	S	S	S	*AAC(3)-I*	5%
	R	R	S	R	S	*AAC(3)-II*	5%
	R	R	S	r	R	*AAC(3)-IV*	44%
	S/r	R	R	R	R	*AAC(6')*	2%
	R	R	S	R	R	*ANT(2')*	5%
	S	S	R	R	S	*APH (3')*	-
Klebsiella spp.	S	S	S	S	S	Classical	-
	R	S	S	S	S	*AAC(3)-I*	3%
	R	R	S	r	S	*AAC(3)-II*	3%
	S/r	R	R	R	R	*AAC(6')*	6%
	R	S	S	R	S	*ANT(2')*	-
	S	S	S	R	R	*APH(3')*	37%
P. aeruginosa	S	S	S	S	S	Classical	-
	R	S	S	R	R	*AAC(3)-I*	-
	R	R	S	R	R	*AAC(3)-III*	81%
	S/r	R	R	R	R	*AAC(6')*	86%
	R	R	S	R	R	*AAC(6')-II*	81%
	R	R	S	R	R	*ANT(2')*	81%
	S	S	S	R	R	*APH(3')*	10%
	R	R	R	R	R	Impermeability	81%

*Classical= Historic phenotype of the species, without acquired resistance
S= Susceptible, R= Resistant, r= reduced zones but likely to remain susceptible at standard breakpoints (Livermore *et. al.,* 2001)
AK-Amikacin, KAN-Kanamycin, GEN-Gentamicin, TOB-Tobramycin and NEO-Neomycin.

Table 4: Presence of Six Aminoglycoside Modifying Enzymes in E. coli, Klebsiella spp., Acinetobacter baumannii and Pseudomonas aeruginosa isolates

AME genes	Amg resistance Conferred	No. tested	Positive M.O for AME genes				PCR product
			E.coli	*Kleb*.spp	*Pseudo*	*A. bau*	
AAC(6')-I	Tob, Amk, Kan	**27**	-	2	5	-	1,100bp
AAC(6')-II	Tob, Gen, Kan	27	1	-	5	-	1,507bp
AAC(6')-1b-cr	Tob, Gen, Kan+ NA, Cip, Nor	37	5	8	7	2	509bp
AAC(3)-I	Gen, Tob	55	2	2	5	-	227bp
AAC(3)-IIa	Gen, Tob	55	6	5	6	-	300bp
ANT(4')-IIb	Tob, Kan	27	-	-	-	-	-

KAN-Kanamycin, AMK- Amikacin, GEN-Gentamicin, TOB-Tobramycin, NA- Nalidixic Acid, CIP-Ciprofloxacin and NOR-Norfloxacin.

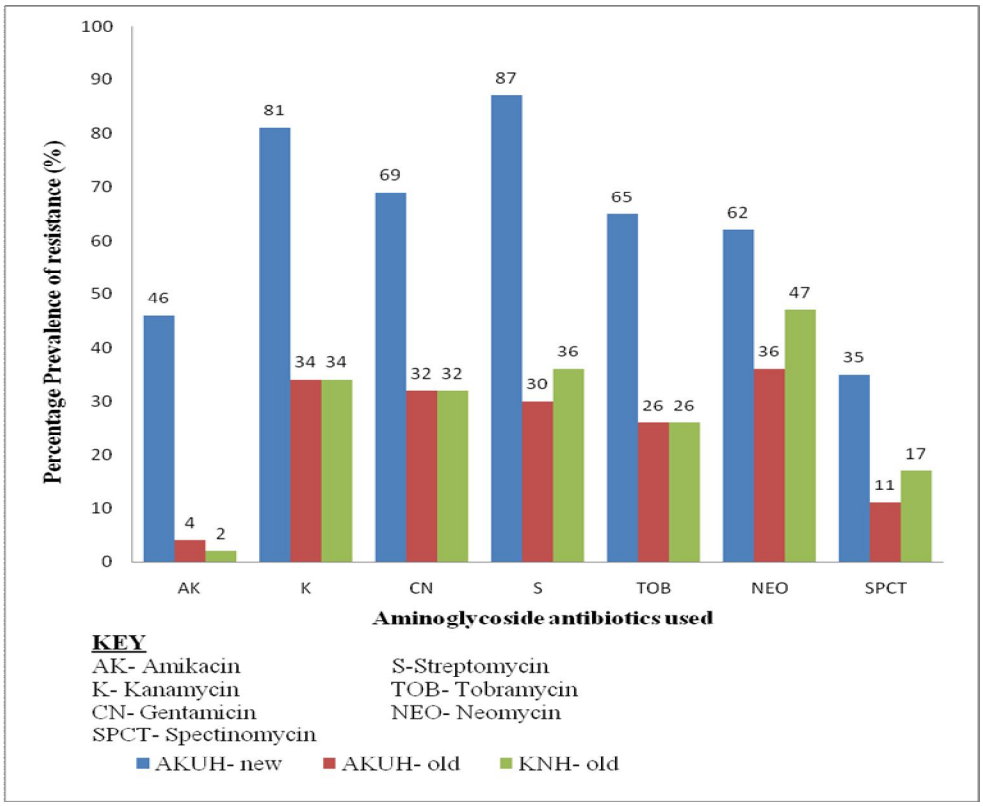

Figure 1: Antibiotic resistance of isolates from Stored KNH-old and AKUH-old laboratory (2001-2006) and AKUH-new (2007-2008) to seven Aminoglycoside antibiotics

Plate 1: Gel photograph of *Aac(6)-Ib-cr* gene PCR products. The resistance gene amplified at 509kb. **Lanes: M-** DNA Marker- Bioline Hyperladder I, 1. *E.coli* 23, 2. *E.coli* 55, 3. *E.coli* 61, 4. *E.coli* 5875, 5. *E.coli* 6303, 6. *E.coli* 6701, 7. *Klebsiella* spp. 2, 8. *Klebsiella* spp. 8, 9. *Klebsiella* spp. 34, 10. *Klebsiella* spp. 6681 11. *Klebsiella* spp 6308,12. *P.aeruginosa* 43, 13. *P. aeruginosa* 63, 14. *P. aeruginosa* 81,15. *P. aeruginosa* 6682,16. *P.aeruginosa* 5876 and 17. *A. baumannii* 5801.

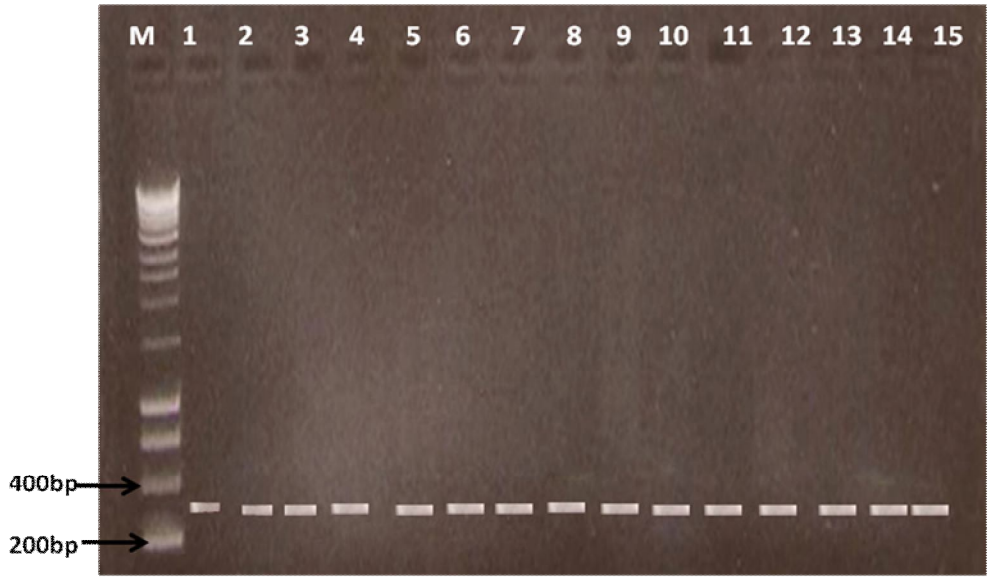

Plate 2: *Aac(3)-IIa* PCR products. The *aac(3)-IIa* gene was amplified at approximately 300bp. **Lanes: M-** DNA Size Marker- Hyper ladder I, 1. *E.coli* 18, 2. *E.coli* 21, 3. *E.coli* 6680, 4. *E.coli* 6698, 5. *E.coli* 6699, 6. *Klebsiella* spp.8, 7. *Klebsiella* spp.9, 8. *Klebsiella* spp. 1667, 9. *Klebsiella* spp. 1671, 10. *Klebsiella* spp. 5422, 11. *P. aeruginosa* 43, 12. *P. aeruginosa* 51, 13. *P. aeruginosa* 64, 14. *P. aeruginosa* 65 and 15. *P. aeruginosa* 5876