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Mutant Prevention Concentrations of Some Aminoglycoside Antibiotics for Fecal Isolates of *Escherichia coli* under different Growth Temperatures

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Abstract
For optimal efficacy, an antibiotic must achieve and sustain at the site of infection, a concentration that can inhibit growth of the bacteria. However, a bacterial infection may contain subpopulations of mutant variants with reduced susceptibility to the antimicrobial agent. There is a great need to periodically evaluate the mutant prevention concentration (MPC) of antibiotic to provide a basis for altering dosing regimens such that the growth of resistant organisms could be curtailed. To evaluate the mutant prevention concentrations (MPCs) of streptomycin, gentamicin and amikacin for fecal *Escherichia coli* isolates under different growth temperatures and determine the extent of recovery of resistant mutants at such temperatures. Fifty (50) isolates of *E. coli* were isolated from stools of patients attending Nasarawa State University Keffi Health Centre in Keffi, Nigeria and identified using standard protocol. Antibiotic minimum inhibitory concentrations (MICs) were determined using macro-broth dilution method of the Clinical and Laboratory Standards Institute (CLSI) with incubation for 24 h at 37°C and 41°C. MIC for 50% (MIC_{50}) and 90% (MIC_{90}) of isolates were then generated from the plot of cumulative frequency curve. MPCs were measured by spreading a series of agar plates containing known aminoglycoside concentrations with approximately 10^{10} CFU of *E. coli* culture and incubated for 48 h at 37°C and 41°C. The lowest aminoglycoside concentration that prevented the growth of resistant colonies was taken as the MPC. MPCs for 50% (MPC_{50}) and 90% (MPC_{90}) of isolates were then generated from the plot of cumulative frequency of the MPCs obtained. MPC/MIC ratios for 50% (MPC_{50}/MIC_{50}) and 90% (MPC_{90}/MIC_{90}) of isolates were also determined. Bacteria surviving (persisting) at MPC were isolated and quantified after 48 h. Statistical analyses of data were done by one-way Analysis of Variance (ANOVA). For each of the drugs, MPC_{50} and MPC_{50}/MIC_{50} were the same at both 37°C and 41°C. MPC_{50} values were: streptomycin (44.2 µg/ml [≥32.0 µg/ml]); gentamicin (44.2 µg/ml [≥32.0 µg/ml]); and amikacin (37.4 µg/ml [≥32.0 µg/ml]); and MPC_{50}/MIC_{50} ratios for each drug at both temperatures were ≤ 3. MPC_{90} and MPC_{90}/MIC_{90} were the same for each drug at both 37°C and 41°C. MPC_{90} values were: streptomycin (253.2 µg/ml [≤256.0 µg/ml]), gentamicin (209.0 µg/ml [≤256.0 µg/ml]), and amikacin (128.0 µg/ml); and MPC_{90}/MIC_{90} ratios for each drug at both temperatures were ≤ 4. Mutant recoveries at the MPCs of the drugs for 50% of the isolates were significantly ($P < 0.05$) different both at 37°C ($P = 0.0089$) and 41°C ($P = 0.0011$). However, mutant recoveries at the MPCs of the drugs for 90% the isolates were insignificantly ($P > 0.05$) different at 37°C ($P = 0.0055$) but significantly ($P > 0.005$) different at 41°C ($P = 0.0080$). Whether at normal body temperature or at a higher body temperature usually obtained during fever, *E. coli* selects and enrich for resistant mutants less easily against streptomycin than gentamicin or amikacin. The extent of recovery of mutants however, is higher at the higher temperature, justifying the common practice of administering high dosage of antimicrobial agent at high body temperature during therapy of bacterial disease.
Keywords: Mutant Prevention Concentration, *Escherichia coli*, Aminoglycoside

1.0 Introduction

Escherichia coli is a common commensal gastro-intestinal tract bacterium found in the large intestine of humans and other warm-blooded animals (Campbell & Reece, 2002); and is responsible for many intestinal and extra-intestinal infections (Bailey et al., 2006). Antimicrobial agents remain the mainstay treatment for infections by *E. coli*; but their continued usefulness in treating infections is being limited by the acquisition of resistance mechanisms (Todar, 2007).

For optimal efficacy, an antibiotic must achieve and sustain at the site of infection, a concentration that can inhibit growth of the bacteria (so-called minimum inhibitory concentration: MIC). However, a bacterial infection may contain subpopulations of mutant variants with reduced susceptibility to the antimicrobial agent. Thus, a therapy effective against the major (susceptible) subpopulation might select for growth of less susceptible mutants. A concentration of antibiotic, termed mutant prevention concentration (MPC) (Dong et al., 1999), which prevents the growth of the least susceptible single-step mutant present in a large bacterial population, was defined. The antibiotic concentration range between MIC and MPC, the mutant selection window (MSW) (Zhao

& Drlica, 2001; Drlica, 2003), is where single-step mutants will be enriched (Baquero & Negri, 1997). By definition, cell growth in the presence of antibiotic concentrations greater than the MPC requires an organism to have developed two or more resistance-causing spontaneous chromosomal point mutations (Dong et al., 2000; Blondeau et al., 2001). The rationale of the MPC concept is to use antimicrobial concentrations above the MSW to restrict selective enrichment of resistant mutants which will improve therapeutic outcome (Drlica & Zhao, 2007).

MPC is a novel concept that has been employed in the evaluation of an antibiotic's ability to minimize or limit the development of resistant organisms (Blondeau et al., 2001). The concept has been developed to provide a basis for altering dosing regimens such that the growth of resistant organisms could be curtailed. The application of this novel concept during antibiotic therapy may have the potential to limit resistance development for organism-antibiotic pairings in which the *in vivo* mechanisms of resistance correspond with those evaluated in *in vitro* MPC studies, i.e. spontaneous point mutations (Smith et al., 2003). However, caution must prevail in the utility of MPC studies conducted on organism-antibiotic pairings in which other mechanisms, such as the presence of inactivating enzymes and efflux, are the primary cause of resistance. The ideal situation for evaluating an MPC requires an organism-antibiotic pairing to have the development of spontaneous chromosomal point mutations as its primary resistance mechanism.

This study evaluates the mutant prevention concentrations (MPCs) of streptomycin, gentamicin and amikacin for fecal *Escherichia coli* isolates under different growth temperatures. The study, in addition, determines the extent of recovery of resistant mutants at such temperatures. To the best our knowledge, no similar study had been undertaken using isolates from this location.

2.0 Materials and Methods

2.1 Bacterial Isolates

A total of 50 fecal *E. coli* isolates were used in this study. They were isolated and identified from stool of patients attending Nasarawa State University Keffi Health Center using standard cultural, microscopical and biochemical procedures. Pink colonies on MacConkey agar (BIOTEC Laboratories Ltd., Ipswich, United Kingdom) that grew with greenish metallic sheen characteristics on eosin methylene blue agar (BIOTEC Laboratories Ltd., Ipswich, United Kingdom) and which were indole positive, methyl red positive, Voges-Proskauer negative and citrate negative were confirmed as *E. coli*. Bacteria were stored in the refrigerator at 4°C on nutrient agar (NA: Merck KGaA, Darmstadt, Germany) slants and reactivated by sub-culturing on MacConkey agar and used in experiments.

2.2 Antibiotics

The antibiotics used were streptomycin (Green Field Pharmaceutical Co. Ltd., China), gentamicin (Yikang Pharmaceutical Co. Ltd., China) and amikacin (Kilitch Drugs Ltd., India). All antibiotics were purchased from the Pharmacy Department, Federal Medical Center, Keffi, Nigeria. The stock solutions were prepared in appropriate solvents in accordance with the CLSI (2011).

2.3 Determination of Minimum Inhibitory Concentration (MIC)

The MICs of streptomycin, gentamicin and amikacin against the *E. coli* isolated from stool and quality control strain (*E. coli* ATCC 25922) were determined in triplicate using the Clinical and Laboratory Standards Institute (CLSI) macro-broth dilution method (CLSI, 2011). An adjusted inoculum of the test organism was inoculated into Mueller-Hinton broth (MHB: BIOTEC Laboratories Ltd., Ipswich, United Kingdom) containing two-fold dilutions of an initial antibiotic solution so that each tube contained approximately 1 x 10^5 colony-forming units (CFU). Results were observed and registered after 24-h incubation at 37°C. MIC was defined as the lowest concentration that inhibited visible growth. Cumulative frequency curves of the antibiotic MICs were plotted; and MICs for 50% (MIC_{50}) and 90% (MIC_{90}) of isolates were then generated from the plots.

2.4 Determination of Mutant Prevention Concentration (MPC)

The MPCs were determined as described elsewhere (Randall et al., 2004) with modifications. Briefly, the tested micro-organisms were cultured in 50 ml of MHB and incubated for 24 h. Then, the suspension was centrifuged (at 4000 g for 10 min) and re-suspended in 10 ml of MHB to yield a concentration of 5 x 10^{10} cfu/10 ml. The inocula were further confirmed through the serial dilution and plating of 100 µl samples on drug-free medium. A series of Mueller-Hinton agar (Fluka Biochemical, Spain) plates containing known concentrations of the aminoglycoside antibiotics were then inoculated with 200 µl each of re-suspended *E. coli* culture (containing approx. 10^{10} cfu). The inoculated plates were incubated for 48 h at 37°C and 41°C, and then screened visually for growth, and colonies counted after the incubation. The MPC was taken as the lowest aminoglycoside concentration that prevents the growth of any mutant after 48 h incubation. All experiments were performed in triplicate. Cumulative frequency curves of the antibiotic MPCs for the isolates were plotted and the MPCs for 50% (MPC_{50}) and 90% (MPC_{90}) of isolates were then generated from the curves. The frequency at which resistant mutant were recovered was calculated as the number of mutants growing in the presence of antibiotic per ml

divided by the inoculum density (1.0 x 10^{10}cfu).

2.5 Statistical Analyses

Mutant recovery for 50% of isolates (MR_{50}) and mutant recovery for 90% of isolates (MR_{90}) were compared at 37°C or 41°C and between temperatures by one way analysis of variance (ANOVA) using Smith Statistical Package (SSP), version 2.80 (by Gary Smith, Pomona College, Claremont, California). Significance of differences was determined at the 5% probability level (that is at P = 0.05).

3.0 Results

3.1 MICs of antibiotics

The minimum and maximum antibiotic MICs (in µg/ml) were (min: max): streptomycin (1.0:128.0), gentamicin (0.25:128.0) and amikacin (1.0:64.0) as shown in Table 1. The MIC_{50} and MIC_{90} of isolates at 37°C and 41°C generated from the cumulative frequency curves in Figure 1 are as shown in Table 1.

3.2 MPCs of antibiotics

The minimum and maximum antibiotic MPCs (in µg/ml) were (min: max): streptomycin (4.0:512.0), gentamicin (2.0:512.0) and amikacin (4.0:512.0) as shown in Table 2. The MPC_{50} and MPC_{90} of isolates at 37°C and 41°C generated from the cumulative frequency curves in Figure 2 are as shown in Table 2. The MPC_{50} of *E. coli* at 37°C and 41°C was the same for streptomycin, gentamicin but slightly lower for amikacin; the MPC_{90} of *E. coli* at 37°C and 41°C was also the same for streptomycin, gentamicin, but lower for amikacin.

3.3 MPC/MIC ratio

The minimum and maximum antibiotic MPC/MIC ratios were (min: max): streptomycin (0.0:8.0), gentamicin (0.0:8.0) and amikacin (0.0:8.0) as shown in Table 3. The antibiotic MPC_{50}/MIC_{50} and MPC_{90}/MIC_{90} ratios for the isolates at 37°C and 41°C generated from the cumulative frequency curves in Figure 3 are as shown in Table 3. The MPC_{50}/MIC_{50} and MPC_{90}/MIC_{90} ratio of streptomycin, gentamicin and amikacin for *Escherichia coli* isolates were the same at 37°C and 41°C. The MPC_{50}/MIC_{50} of streptomycin, gentamicin and amikacin were $\leq$ 4.0 and MPC_{90}/MIC_{90} of streptomycin, gentamicin and amikacin were $\leq$ 4.0 as shown in Table 3. A lower value of the MPC/MIC ratio indicates a better ability to prevent the emergence of mutants.

3.5 Mutant Recovery

The mutant recovery (in percentage) at MPC_{50} and at MPC_{90} of streptomycin, gentamicin and amikacin for *E. coli* at 37°C and 41°C is as shown in Table 4. Generally more mutants were recovered at higher temperature for all the aminoglycosides tested, although the difference was only significant in the case of amikacin as shown in Table 5. Differences in mutant recovery between the aminoglycosides at both 37°C and 41°C were significant.

3.6 Statistical Analyses

The mutant recovery at MPC_{50} (MR_{50}) of *E. coli* isolates for streptomycin, gentamicn, and amikacin at 37°C were compared with those at 41°C as shown in Table 5. Similarly, mutant recovery at MPC_{90} (MR_{90}) for *E. coli* isolates for streptomycin, gentamicin and amikacin at 37°C were also compared with those at 41°C.

4.0. Discussion

Traditional dosing of antimicrobial agent is based on the MIC. Within this MIC, the susceptible subpopulation are suppressed and the less susceptible subpopulation persist (Craig, 2002; Roberts et al., 2008). The concentration of antimicrobial agent that can prevent the growth of both the susceptible subpopulation and the less susceptible subpopulation known as Mutant Prevention Concentration (MPC) can prevent selection and enrichment of resistant mutant. If an antibiotic concentration is maintained above the MPC, resistant bacteria should not be selected (Drlica, 2003). MPC concept can help in making decision on dosing regimen of antimicrobials with respect to the potential for the selection and enrichment of resistant mutant (Ambrose et al., 2002; Tam et al., 2005).

This study reports the MPCs, MPC/MIC ratios and mutant recovery at MPC of streptomycin, gentamicin and amikacin for fecal isolates of *E. coli* under normal body (37°C) and elevated (41°C) temperatures. The similarity observed in the MPC_{50} of each aminoglycoside antibiotic at both 37°C and 41°C suggest that a rise in body temperature experienced during fever might not affect the concentration of antimicrobial agent that can prevent selection and enrichment of resistant mutant. Since aminoglycoside resistance is primarily attributed to the presence of inactivating enzymes that are acquired (French & Phillips, 1997; Fluit et al., 2001), MPCs that are obtained during studies with aminoglycosides do not accurately reflect *in vivo* resistance mechanisms which occur in the clinical setting. However, since MPC measures the *in vitro* susceptibility of an antibiotic exposed to higher numbers of bacteria (~10^9-10^{10} cfu), closer to what is found in real infections, it presents a more realistic susceptibility assessment tool than MIC, where the standardized inoculum tested is ~10^5 cfu. MPC studies of aminoglycosides have been conducted with organisms including *Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter cloacae*, *E. coli*, *Klebsiella pneumoniae*, *Pseuodonas aeruginosa*, *Staphylococcus aureus* and *Stenotrophomonas maltophilia* (Akins et al., 2002a; Akins et al., 2002b; Zhao & Drlica, 2002).

The lower MPC/MIC ratio for streptomycin observed in this study, compared with gentamicin and amikacin, suggests streptomycin as less prone to the emergence of mutants.
The relatively higher mutant recovery at 41°C suggests that the selection and enrichment of resistance mutants can be encouraged by a rise in temperature as usually observed during fever. This observation agrees with a study with *E. coli* on the recovery of resistance mutants against fluoroquinolones under different condition like aerobiosis, anaerobiosis and temperature at 27°C and 37°C (Linde & Lehn, 2004).

5.0. Conclusion

In conclusion, amikacin has the least MPC. In addition, whether at normal body temperature or at a higher body temperature usually obtained during fever, *E. coli* selects and enrich for resistant mutants less easily against streptomycin than gentamicin or amikacin. Furthermore, the extent of recovery of mutants however, is higher at the higher temperature, providing a basis for administering high dosage of these agents at high body temperature during therapy of bacterial disease. MPC measurement of aminoglycoside susceptibility can thus be used to predict and prevent the evolution of resistance, which should be a parallel goal with curing the infection itself.

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Table 1: Minimum Inhibitory Concentration of some aminoglycosides for 50% and 90% of fecal *Escherichia coli* isolates at 37°C and 41°C

Antibiotics	MIC Ranges (µg/ml)	Minimum Inhibitory Concentration (µg/ml)	
		MIC_{50}	MIC_{90}
Streptomycin	1.0-128.0	25.4 (≤ 32.0)	65.4 (≤ 128.0)
Gentamicin	0.25-128.0	15.9 (≤ 16.0)	62.7 (≤ 64.0)
Amikacin	1.0-64.0	13.5 (≤ 16.0)	34.5 (≤ 64.0)

MIC_{50} = Minimum Inhibitory Concentration for 50% of isolates; MIC_{90} = Minimum Inhibitory Concentration for 90% of isolates.

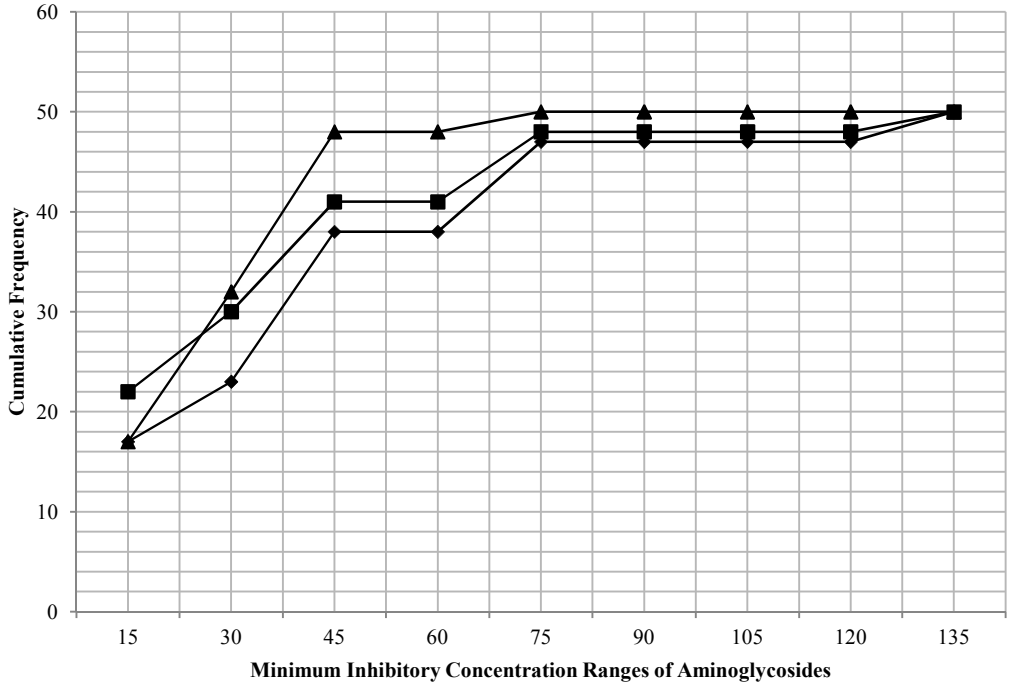

Figure 1: Cumulative Frequency Curves of aminoglycoside MICs for isolates of *Escherichia coli* (Streptomycin, Gentamicin, Amikacin).

Table 2: Mutant Prevention Concentration of some aminoglycosides for 50% and 90% of fecal *Escherichia coli* isolates at 37°C and 41°C

Antibiotics	MPC Ranges (µg/ml)	Mutant Prevention Concentration (µg/ml)	
		MPC_{50}	MPC_{90}
Streptomycin	4.0-512.0	44.2 (≤ 64.0)	253.2 (≤ 256.0)
Gentamicin	2.0-512.0	44.2 (≤ 64.0)	209 (≤ 256.0)
Amikacin	4.0-512.0	37.4 (≤ 64.0)	128.0

MPC_{50} = Mutant Prevention Concentration for 50% of isolates; MPC_{90} = Mutant Prevention Concentration for 90% of isolates.

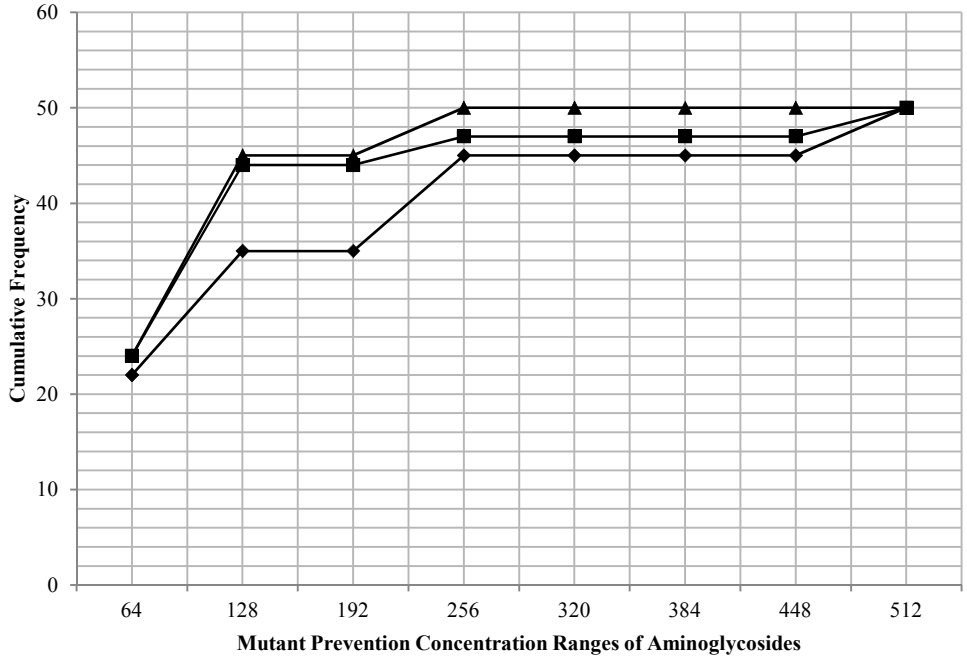

Figure 2: Cumulative Frequency Curves of aminoglycoside MPCs for isolates of *Escherichia coli* (Streptomycin, Gentamicin, Amikacin).

Table 3: Mutant Prevention Concentration/Minimum Inhibitory Concentration ratio of some aminoglycosides for 50% and 90% of fecal *Escherichia coli* isolates at 37°C and 41°C

Antibiotics	**MPC/MIC Ranges**	**Mutant Prevention Concentration/ Minimum Inhibitory Concentration**	
		MPC_{50}/MIC_{50}	**MPC_{90}/MIC_{90}**
Streptomycin	0.0-8.0	1.7 (≤ 4.0)	3.9 (≤ 4.0)
Gentamicin	0.0-8.0	2.8 (≤ 4.0)	3.3 (≤ 4.0)
Amikacin	0.0-8.0	2.8 (≤ 4.0)	3.7 (≤ 4.0)

MPC_{50}/MIC_{50} = Mutant Prevention Concentration/Minimum Inhibitory Concentration ratio for 50% of isolates;
MPC_{90}/MIC_{90} = Mutant Prevention Concentration/Minimum Inhibitory Concentration ratio for 90% of isolates.

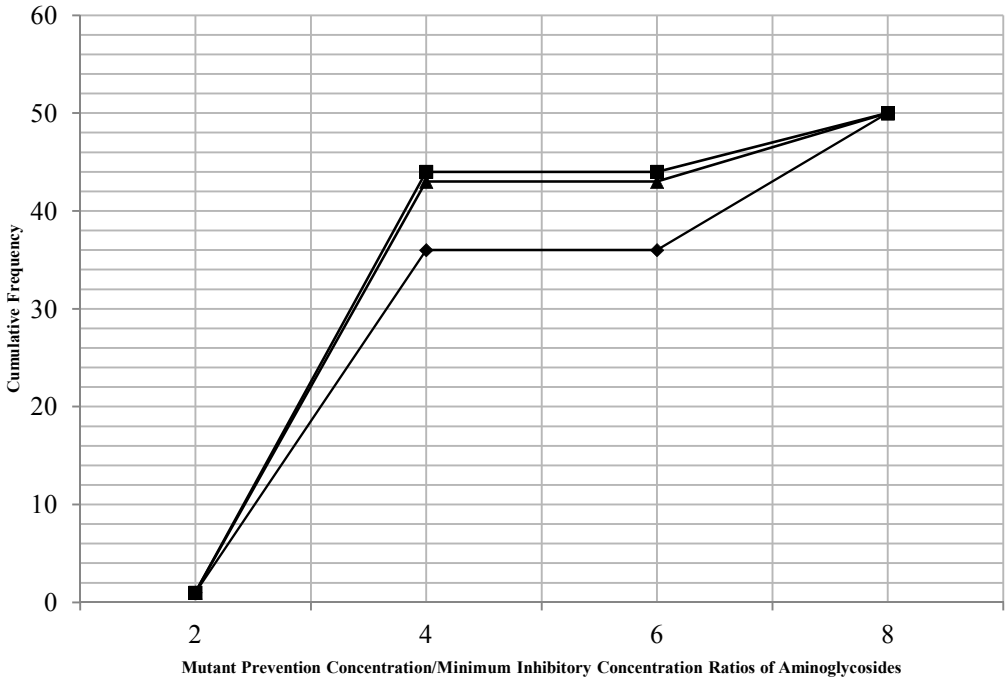

Figure 3: Cumulative Frequency Curves of aminoglycoside MPC/MIC ratios for isolates of *Escherichia coli* (◆ Streptomycin, ■Gentamicin, ▲ Amikacin).

Table 4: Mutant recovery (%) at MPC_{50} and MPC_{90} of some aminoglycosides for fecal *Escherichia coli* isolates at 37°C and 41°C

Antibiotics	**Mutant recovery (%) at MPC_{50} (MR_{50})**		**Mutant recovery (%) at MPC_{90} (MR_{90})**	
	37°C	41°C	37°C	41°C
Streptomycin	$6.0 \times 10^7 \pm 1.30$	$7.6 \times 10^7 \pm 2.00$	$6.0 \times 10^7 \pm 2.00$	$6.0 \times 10^7 \pm 2.00$
Gentamicin	$6.6 \times 10^7 \pm 8.00$	$8.2 \times 10^7 \pm 6.00$	$7.4 \times 10^7 \pm 1.20$	$8.7 \times 10^7 \pm 3.00$
Amikacin	0 ± 0.00	0 ± 0.00	$4.6 \times 10^7 \pm 1.00$	$5.8 \times 10^7 \pm 2.00$

MR_{50} = Mutant recovery at MPC_{50}; MR_{90} = Mutant recovery at MPC_{90}.

Table 5: Statistical analyses of mutant recovery at MPC_{50} and MPC90 of some aminoglycosides for fecal *Escherichia coli* isolates at 37°C and 41°C

Statistics	**P value**	**Remarks (at P = 0.05)**
MR_{50} Streptomycin (37°C vs. 41°C)	0.1573	Insignificant
MR_{50} Gentamicin (37°C vs. 41°C)	0.2566	Insignificant
MR_{50} Amikacin (37°C vs. 41°C)	0.0000	Significant
MR_{90} Streptomycin (37°C vs. 41°C)	0.1982	Insignificant
MR_{90} Gentamicin (37°C vs. 41°C)	0.1215	Insignificant
MR_{90} Amikacin (37°C vs. 41°C)	0.1153	Insignificant
MR_{50} for all the drugs (at 37°C)	0.0089	Significant
MR_{50} for all the drugs (at 41°C)	0.0011	Significant
MR_{90} for all the drugs (at 37°C)	0.0055	Significant
MR_{90} for all the drugs (at 41°C)	0.0080	Significant

MR_{50} = Mutant Recovery for 50% of isolates; MR_{90} = Mutant Recovery for 90% of isolates.

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