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In vitro Induction of Phenotypic Resistance to Antibiotics in some Pathogenic Bacteria

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Abstract
The study was carried out on the development of resistance due to repeated exposure of some bacterial isolates to antibiotics. The organisms; *Salmonella typhi* and *Shigella dysentriae* were isolated from stools using Salmonella Shigella Agar (SS Agar). *Proteus mirabilis* and *Staphylococcus aureus* were isolated from urine using Cystine Lysine Electrolite Deficient (CLED) Agar. After the isolation, standard inoculum of *Salmonella typhi,Shigella dysentriae, Proteus mirabilis* and *Staphylococcus aureus* were prepared, each and was streaked onto Mueller-Hinton Agar plates. Prepared Amoxicillin, Ciprofloxacin, and Gentamicin paper discs were placed each at the center of the plates and incubated for 24 hours, at 37^{o}C. Zones of inhibition were formed. The zones of inhibition were measured and recorded, and then bacteria from the edges of the inhibition zones were picked up with a swab stick, and inoculated on to fresh Mueller-Hinton Agar plates. This process was repeated of 10 timesfor each. Over the course of 10 exposures to test antibiotics separately, all the test organisms developed resistance to the antibiotics gradually as seen through decrease in diameter of their zones of inhibitions. *Salmonella typhi* plates (from 1-10 exposure) under Ciprofloxacin, on average were reduced to 24.1mm, Gentamycin were reduced to 6.5mm and Amoxicillin were reduced to 5.8mm. *Shigella dysentriae* plates (from 1-10 exposure) under Ciprofloxacin, on average were reduced to 27.2mm, Gentamycin were reduced to 7.8mm and Amoxicillin were reduced to 6.0mm. *Proteus mirabilis* plates (from 1-10 exposure) under Ciprofloxacin, on average were reduced to 34.0mm, Gentamycin were reduced to 22.7mm and Amoxicillin were reduced to 8.5mm. *Staphylococcus aureus* plates (from 1-10 exposure) under Ciprofloxacin, on average were reduced to 25.9mm, Gentamycin were reduced to 15.4mm and Amoxicillin were reduced to 7.4mm. The results obtained confirmed that repeated exposure of the bacterial pathogens to antibiotics increased their resistance. Ciprofloxacin was the most active antibiotic among the test antibiotics as it has notable zone of inhibition often repeated exposure while Amoxicillin was the least active antibiotic as it showed full resistance at 4th exposure for *Salmonella typhi* and *Shigella dysenteriae* and 5th exposure for *Proteus mirabilis* and *Staphylococcus aureus*.
Keywords: Antibiotics, Development of Resistance, Repeated Exposure

INTRODUCTION

Antibiotic resistance is a serious and growing global problem. It occurs when bacteria change in some way that reduces or eliminates the effectiveness of drugs, chemicals, or other agents designed to cure or prevent infections. The bacteria survive and continue to multiply causing more harm. Bacteria can do this through several mechanisms. Some bacteria develop the ability to neutralize the antibiotic before it can do harm, others can rapidly pump the antibiotic out, and still others can change the antibiotic attack site so it cannot affect the function of the bacteria (WHO, 2014).Some organisms are naturally resistant but the term most often refers to acquired resistance, which can be a result of either new mutations or transfer of resistance genes between organisms (Woodford and Ellington, 2007). The increasing rates of antibiotic resistant microbes are caused by antibiotic use from human and veterinary medicine. Any use of antibiotics can increase selective pressure in a population of bacteria, promoting resistant bacteria and causing vulnerable bacteria to die (Leekha *et al.*, 2011).

Antibiotics kill or inhibit the growth of susceptible bacteria. Sometimes one of the bacteria survives because it has the ability to neutralize or escape the effect of the antibiotic; that one bacterium can then multiply and replace all the bacteria that were killed off (Cassir *et al.*, 2014).

Exposure to antibiotics therefore provides selective pressure, which makes the surviving bacteria more likely to be resistant. In addition, bacteria that were at one time susceptible to an antibiotic can acquire resistance through mutation of their genetic material or by acquiring pieces of DNA that code for the resistance properties from other bacteria. The DNA that codes for resistance can be grouped in a single easily transferable package. This means that bacteria can become resistant to many antimicrobial agents because of the transfer of one piece of DNA (Hoffman *et al.,* 2015).

Several molecular mechanisms of antibacterial resistance exist. Intrinsic antibacterial resistance may be part of the genetic makeup of bacterial strains (Alekshun, 2007). For example, an antibiotic target may be absent from the bacterial genome. Acquired resistance results from a mutation in the bacterial chromosome or the acquisition of extra-chromosomal DNA. Antibacterial-producing bacteria have evolved resistance mechanisms that have been shown to be similar to, and may have been transferred to, antibacterial-resistant strains (Nikaido, 2009). The spread of antibacterial resistance often occurs through vertical transmission of mutations during growth and by genetic recombination of DNA by horizontal genetic exchange. For instance, antibacterial resistance genes can be exchanged between different bacterial strains or species via plasmids that carry these resistance genes (Witte, 2004). Plasmids that carry several different resistance genes can confer resistance to multiple antibacterial drugs. Cross-resistance to several antibiotics may also occur when a resistance mechanism encoded by a single gene conveys resistance to more than one antibacterial compound (Baker-Austin *et al.*, 2006). The aim of this work is to develop phenotypic resistance by pathogenic bacteria due repeated exposure to antibiotics.

MATERIALS AND METHODS

Isolation and identification of Bacteria

Isolation of *Salmonella typhi* and *Shigella dysentriae* From Stool

Clean, dry, sterile, disinfectant-free suitable wide-necked containers were used to collect the stool samples from patients. The patients were asked to avoid contaminating the faeces with urine (Cheesbrough, 2006). When the specimen was formed or semi-formed, a thick suspension of it was made in about 1 ml of sterile Peptone water. A loopful of fresh emulsified faeces or a fluid specimen was inoculated on *Salmonella Shigella* (SSA) Agar. The SS Agar plate was incubated aerobically at 37°C overnight. *Salmonella* produced colorless colonies 1–2 mm in diameter with black center, while *Shigella* produced colourless colonies, 2-4 mm in diameter without black center. *Salmonella typhi* and *Shigella dysentriae* were identified by urease test, Indole test, Methyl Red test, Voges Proskaeur Test, Motility test, Triple sugar iron agar (TSI) test, Oxidase test, Citrate test, Lactose test, Mannitol test and H_2S production (Cheesbrough, 2006).

Isolation of *Proteus mirabilis* and *Staphylococcus aureus* from Urine

Sterile, dry, leak-proof containers were used to collect samples from patients. Clean-catch specimen was mixed by rotating the container. Using a sterile wire loop (one that holds 0.002 ml), a loopful of urine was inoculated on a quarter plate of CLED (Cystine Lactose Electrolyte-Deficient) Agar. The plate was incubated aerobically at 35–37 °C overnight (Cheesbrough, 2006).

Proteus mirabilis produced blue –gray translucent colonies while *Staphylococcus aureus* produced deep yellow colonies of uniform colour. *Staphylococcus aureus* which was coagulase positive was confirmed by coagulase test, Catalase test, mannitol test, oxidase test and blood Hemolysis test. *Proteus mirabilis* was confirmed by urease test, oxidase test, indole test, citrate test, Methyl Red test, Voges Proskaeur Test, Motility test, Triple sugar iron agar (TSI) test, H_2S production test (Cheesbrough, 2006).

Preparation of Turbidity Standard (Equivalent to 0.5 McFarland Standards)

A 1% v/v solution of sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to99 ml of water and mix well. A 1% w/v solution of barium chloride was also prepared by dissolving 0.5g of dihydrate barium chloride ($BaCl_2.2H_20$) in 50 ml of distilled water. 0.6 ml of the barium chloride solution was added to 99.4 ml of the sulphuric acid solution, and mix, a small volume of the turbid solution was transfer to a capped tube bottle which was used as standard turbidity (Oyeleke and Manga, 2008). Using a sterile wire loop, colonies were emulsified in 2ml of sterile physiological saline and standard turbidity was obtained.

Inoculation of Samples

Mueller-Hinton Agar was prepared and autoclaved according to manufacturer. The media was poured into Petri dishes to depth of 4mm (about 20ml per plate). Care was taken to pour the plates on a level surface so that the depth of the medium was uniform.

After the gel had solidified, using a sterile swab, the standard inoculum of *Salmonella typhi, Shigella dysentriae, Proteus mirabilis and Staphylococcus aureus* prepared, each was streaked onto the Muller Hinton agar plates (Cheesbrough, 2006).

Exposure to Antibiotics

Prepared Amoxicillin, Ciprofloxacin, and Gentamicin paper discs were placed at the center of the plates and the plates were incubated for 24 hours, at 37^0C. The bacteria were confirmed sensitive to the antibiotics, a zone of inhibition was formed. The zones of inhibition were measured and recorded, and then bacteria from the edges of the inhibition zones were picked up with a swab sticks, and inoculated on to new Mueller-Hinton Agar plates. This process was repeated for each of 10 days exposures (Cheesbrough, 2006).

RESULTS

Repeated Exposure of Bacteria to Antibiotics

Over the course of repeated exposure to the antibiotics, all the bacteria developed and gained resistance to the antibiotics gradually. The following results were obtained as shown in tables 1.0 to 4.0.

Salmonella typhi exposed to Ciprofloxacin, Gentamycin and Amoxicillin Table 4.1 (*Salmonella typhi*) showed sensitivity values to gradually decreased, from 1st to 10th exposure and was observed to be from 44mm to 15mm. Gentamycin decreasedfrom 27mm at 1st exposure to18mm at 3rd exposure. Amoxicillin also shows gradual decrease of zone diameter, which is from 23mm to 15mm, at 1st to 3rd exposure respectively as shown in Table 1.

Table 1: Zone of Inhibition of *Salmonella typhi* after Repeated Exposure to Antibiotics

	Zone diameter (mm)									
Antibiotics/ Exposure days	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th Day	10th day
Ciprofloxacin	44	38	35	30	25	23	20	17	17	15
Gentamycin	27	20	18	00	00	00	00	00	00	00
Amoxicillin	23	20	15	00	00	00	00	00	00	00

Shigella disenteriae isolated from stool samples collected from different patients was repeatedly exposed to Ciprofloxacin, Gentamycin and Amoxicillin Table 2 (*Shigella dysenteriae*) showed the antibiotic, sensitivity test for each test antibiotic, Ciprofloxacin zones gradually decreased, from 1st to 10th exposure was observed as 35mm to 20mm respectively, for Gentamycin decreased of zone diameter was from 26mm at 1st exposure to 15mm at 4th exposure. Amoxicillin also shows gradual decrease of zone diameter, which is from 25mm to 15mm, at 1st to 3rd exposure respectively as shown in Table 2.

Table 2: Zone of Inhibition of *Shigella dysentriae* after Repeated Exposure to Antibiotics

	Zone diameter (mm)									
Antibiotics/ Exposure days	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th Day	10th day
Ciprofloxacin	35	35	30	28	28	27	24	23	22	20
Gentamycin	26	22	15	15	00	00	00	00	00	00
Amoxicillin	25	20	15	00	00	00	00	00	00	00

Proteus mirabilis isolated from urine samples collected from different patients was repeatedly exposed to Ciprofloxacin, Gentamycin and Amoxicillin. Table 3 (*Proteus mirabilis*) showed the antibiotic sensitivity test, Ciprofloxacin zone gradually decrease, from 1st to 10th exposure was observed as 44mm to 25mm respectively, for Gentamycin decreased of zone diameter was observed from 30mm at 1st exposure to 15mm at 10th exposure. Amoxicillin also shows gradual decrease of zone diameter, which is from 25mm to 15mm, at 1st to 4th exposure respectively as shown in Table 3.

Table 3: Zone of Inhibition of *Proteus mirabilis* after Repeated Exposure to Antibiotics

	Zone diameter (mm)									
Antibiotics/ Exposure days	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th Day	10th day
Ciprofloxacin	44	44	44	40	30	30	28	28	27	25
Gentamycin	30	30	24	24	23	23	20	20	18	15
Amoxicillin	25	25	20	15	00	00	00	00	00	00

Staphylococcus aureus isolated from urine samples collected from different patients was repeatedly exposed to Ciprofloxacin, Gentamycin and Amoxicillin; Table 4 (*Staphylococcus aureus*) showed the antibiotic sensitivity test, Ciprofloxacin zone gradually decreased, from 1st to 10th exposure was observed as 35mm to 18mm, respectively, for Gentamycin decreased of zone diameter was observed from 30mm at 1st exposure to 17mm at 7th exposure. Amoxicillin also shows gradual decrease of zone diameter, which is from 22mm to 15mm, at 1st to 4th exposure as shown in Table 4

Table 4: Zone of Inhibition of *Staphylococcus aureus* after Repeated Exposure to Antibiotics

	Zone diameter (mm)									
Antibiotics/ Exposure days	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th Day	10th day
Ciprofloxacin	35	35	32	30	25	22	22	20	20	18
Gentamycin	30	29	25	22	19	15	14	00	00	00
Amoxicillin	22	20	17	15	00	00	00	00	00	00

DISCUSSION

The results obtained confirmed that repeated exposure of the bacterial pathogens increased their resistance to the antibiotics they were exposed to as reported by James (2015). This means that the bacteria developed resistance when repeatedly exposed to a particular antibiotic. This could be due to the following reasons: Antibiotic modification; bacterial enzymes like *Beta lactamase* alter the structure of the antibiotic and thereby render the antibiotic ineffective. The mechanism of resistance in Gram positive and negative bacterial species to *Beta lactam* antibiotics, by preventing the antibiotic from entering the bacterial cell or pumping it out quicker than it floods in. Antibiotic is unable to inhibit the activity of the target structure in the bacteria because of structural changes in the bacterial molecule. The bacteria produced an alternative target like an enzyme that is resistant to inhibition by the antibiotic while continuing to produce the original sensitive target. This allows the bacteria to survive in the face of selection as reported by Hawkey (1998) or as result of mutation in the bacterial chromosome or the acquisition of extra-chromosomal DNA as reported by Alekshun (2007).

It is also observable from the results that Ciprofloxacin was the most active antibiotic against the test organism as it has notable zone of inhibition in all the organisms upon repeated exposure. This was due to its very good spectrum of activity against several clinically important aerobic Gram negative bacilli like those belonging to *Enterobacteriaceae* (eg *E coli*) and *Pseudomonas aeruginosa*. They are also active against Gram positive cocci like *S pneumoniae*, *S aureus* and beta haemolytic *streptococci.H influenzae*, *Chlamydia pneumoniae*, and *Mycoplasma pneumonia* (Velissariou 2006). While Amoxicillin was the least active antibiotic against the test bacteria with exhibition of full resistance on 4th exposure for *shigella dysenteriae* and *Salmonella typhi* and on 5th exposure for *Proteus mirabilis* and *Staphylococcus aureus*. This could be due to the ability of bacteria to produce ß-lactamase when encountered with ß-lactam antibiotics as reported by (Allen *et al.*, 2009).

The implications of antibiotic resistance is that many of the available treatment options for common bacterial infections are becoming more and more ineffective. As a consequence, there are situations where infected patients cannot be treated adequately by any of the available antibiotics. This resistance may delay and hinder treatment, resulting in complications or even death. Moreover, a patient may need more care, as well as the use of alternative and more expensive antibiotics, which may have more severe side effects, or may need more intensive treatments, such as intravenous injection, to be given in hospitals according to (WHO, 2014).

Exposure of micro flora to antibiotics may increase the number of resistant factors which can transfer resistance to pathogenic bacteria (Mathew *et al.*, 2007). There is a strong association between consumption of antibiotic and antibiotic resistance of bacteria. It is evident-based with the B-lactamases. Horizontal gene transfer (HGT) has a main role in the progress and diffusion of the resistance to the B-lactam antibiotic among the enteric bacteria in both community and hospital level infections. Regular mutations in the genome of DNA create resistance to Fluoroquinolones and other antibiotics by transfer of DNA between bacterial strains (Davies and Davies, 2010).

The findings in this research are in line with the findings of James (2015). who confirmed that repeated exposure leads to antibiotic resistance in some bacteria.

This was supported by Betty *et al,* (1993) who stated that repeated and improper uses of antibiotics are primary causes of the increase in drug-resistant bacteria. The results obtained also conforms with the findings of (Sule *et al.,* 2002) which specified that floroquinolones are very effective against most of the bacteria which are resistant to other antibiotics.

CONCLUSION

The results obtained confirmed that repeated exposure could lead to antibiotic resistance in *Salmonella typhi, Shigella dysenteriae, Proteus mirabilis and Staphylococcus aureus*. The results also confirmed that Ciprofloxacin is the most effective antibiotic against the test organism as it has notable zone of inhibition in all the organisms upon repeated exposure.

RECOMMENDATIONS

In view of the results obtained, it is strongly recommend that:

Antibiotics should only be administered to patients if proven to be effective through antibiotics sensitivity testing.

The same drug should not be repeatedly administered for the treatment of a particular disease in case of reinfection.

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