NISEB JOURNAL Vol. 11, No. 4, October 31, 2011
Printed in Nigeria

1595-6938/2011 $5.00 + 0.00

http://www.nisebjournal.org

NISEB 2011100/11403

Fluoroquinolone efflux pump of *Pseudomonas aeruginosa*

I. J. Ehiaghe[1], F. A. Ehiaghe*[2], B. O. Akinshipe[3] and R. Ilobanafor[4]

[1]Lahor Research Laboratory, Benin City, Edo State, Nigeria
08060138954, Fredleo2547@yahoo.com

[2]Department of Haematology, Igbinedion University, Okada, Edo State, Nigeria
08063327432, Fredleo2547@yahoo.com

[3]Department of Medical Microbiology, Igbinedion University, Okada, Edo State, Nigeria
08056736595, Bensonakinshipedr01@yahoo.com

[4]Department of Medical Laboratory Science, Igbinedion University, Okada, Edo State, Nigeria
08038679483, ginacaeli@ovi.com

(Received May 20, 2011; Accepted July 10, 2011)

ABSTRACT: A total of thirty six (36) *Pseudomonas aeruginosa* were isolated from clinical specimens (ear swab, urine, wound swab, sputum and semen) in Benin City. Fourteen (14) shows resistance to Ciprofloxacin, seventeen (17) to Ofloxacin, sixteen (16) to Perfloxacin and twenty (20) to Sparfloxacin after the first susceptibility was performed. The post curing antibiogram of the multidrug resistant clinical isolates were retested against the FQs. *Pseudomonas aeruginosa* showed 64% resistance to Ciprofloxacin, 88% to Ofloxacin, 87.5% to Perfloxacin and 85% to Sparfloxacin. Hence FQs resistance to this organism is more likely due to mutation of the target site or altered DNA gyrase and efflux pump. Also, plasmid analysis was carried out on clinical isolate that shows significant plasmid DNA mediation with uniform size indicating that such plasmid mediation have a similar origin and organisms are closely related. Base on this study, resistance rate were low with ciprofloxacin, thus this drug remain the rational choice for empirical treatment of serious infection as mutation, efflux pump and other factors should be taken into consideration when manufacturing newer Fluoroquinolones antimicrobial regimen for the treatment of Pseudomonas.

Key word; Efflux pump, Fluoroquinolones, *Pseudomonas aeruginosa*.

Introduction

The genus *Pseudomonas* is non-fermentative but utilizes sugars oxidatively and grows aerobically. The genus comprises more than 140 species and the most important species pathogenic to man is *Pseudomonas aeruginosa*. Other species are saprophytic and occur widely in nature (Kohler, 1997). *Pseudomonas aeruginosa* – sometimes colonizes humans and is the major human pathogen, produces infections in patient with abnormal host defense and is an important nosocomial pathogen (Henderson, 1988).

*Author to whom all correspondence should be addressed.

Morphologically, *Pseudomonas aeruginosa* is motile, rod shape, measuring about 0.6 x 2Nm. It is gram negative and occurs as single bacteria in pairs and occasionally in short chains grow widely in many types of culture media at 37^{o}C - 42^{o}C and its growth widely at 42^{o}C helps to differentiate it from other pseudomonas species. It is oxidase positive (Bodey, 1983).

Active efflux of toxic compound in cells is a general mechanism that bacteria have developed to protect themselves against the adverse effects of their environments. Antibiotic that are used in clinical setting are among these toxic compounds and extrusion of them from bacterial cells significantly decreases there clinical utility (Jones *et al*., 1980).

Antibiotics are expelled from the cells by membrane transporters proteins, the so called drug efflux pumps and of particular interest are efflux pumps capable of extruding out of the cell a large variety of structurally unrelated compound with different bacterial modes of action (Lawrence and Barret, 2004).

Most of the genes encoding these Multi-drug Resistance (MDR) pumps are normal constituents of bacterial chromosomes. Some of these genes have a relatively high level of constitutive expression and confer so-called intrinsic resistance to antibiotics. In Gram-negative bacteria, most of the efflux pumps that contribute to both intrinsic and acquired resistance to clinically useful antibiotics are three components. Structure that inverse both inner membranes and outer membranes. Each tripartite pump contains a transport located in the cytoplasm membrane, an outer membrane channel and a linker protein, which is thought to bring the other two components into contact. The structural organization allows extrusion of substrates directly into the external medium by passing the periplasmic. (Poole, 2002).

Objectives of the study

1. To investigate antimicrobial resistance conferred in isolates of *Pseudomonas aeruginosa* to some fluoroquinolones by efflux pump.
2. To attempt curing the various *Pseudomonas aeruginosa.*
3. To determine the plasmid profile of multi-drug resistant *Pseudomonas aeruginosa* this includes checking the size of such plasmid.

The resistance of *Pseudomonas aeruginosa* to these fluoroquinolones under study will be checked if they are chromosomal or plasmid mediated as a result of the efflux pump. So that when new antibiotic are produce, efflux mechanism should be put into consideration in order to avoid chemotherapeutic failure of clinical isolate of *Pseudomonas aeruginosa*

Materials and Methods

Study Area

Samples were collected from Central Hospital Management Board and some private laboratories (including Lahor Research Laboratory) within Benin City. This sample include specimen of ear swab, wound swab, mid stream urine, sputum and semen.

Sample Size and Collection

One hundred and fifty samples were collected from the various specified laboratories, and out of the 150 samples 36 were *Pseudomonas spp.* stored on nutrient agar slant of Marcartney bottles.

During the course of this study the following materials were used: Standard wire loop, MacConkey agar, Blood agar, Nutrient agar, Meuller hinton agar, Nutrient agar slants, Automatic pipette, Swab stick, Universal container, Autoclave, Incubator, Sodium dodecyl sulphate, Eppendorf tubes, Antibiotic discs, UV Trans illumination, 0.8% agarose gel, Microwave, Ethidium bromide, Electrophoretic tank, Buffers (1A, 2B & 3C), 70% ethanol, Hot air oven, Pipette, Centrifuge, Weighing balance (electronic), UV light. All the media were prepared according to the manufacturer's specification.

Culture Methods

With a grease pencil, the bottom of the various plates were divided into 3 parts, then the samples were cultured on the media and incubated at 37^{o}C for 24 to 48hrs depending on the nature of the sample.

Isolation and Identification

The isolates were identified by standard techniques as described by (Cowan and Steel, 1974) which included colonial appearance of the organism on the media, morphological characteristics such as size, form, elevation, opacity, colour, odour and edge. Specific biochemical tests were performed for identification of the organisms. However, prior to this, films were made from the colonies and stained by Gram's techniques, which acted as a guide to the possible identity of the organisms. After the primary identification of the Gram-negative organism, were further subjected to specific biochemical tests such as citrate utilization, indole production, urease production, oxidase test, motility test and sugar fermentation (sucrose, salicin, lactose, maltose and mannitol) test.

Treatment of Isolates

Each *Pseudomonas aeruginosa* was inoculated into nutrient agar slant and incubated at 37^{o}C for 24hrs, after which each nutrient agar slant was sub cultured onto MacConkey agar plate to get discrete colonies for susceptibility test.

Antibiotic Sensitivity Testing

Sensitivity tests were performed on each *Pseudomonas aeruginosa* isolate after sub-cultured from the nutrient agar slant onto MacConkey agar were cultured in Mueller hinton agar to determine its susceptibility pattern to antibiotic using the disc diffusion method (Clinical Laboratory Standard Institute, 2007). The sensitivity of the test organisms to a particular antibiotic e.g. (Cpx, ofl, pef & spx) was scored by measuring the zone of inhibition. Zones of inhibition less than 13mm were regarded as resistant while those measuring 15mm and above as sensitive. (Pre-antibiogram).

Thereafter, the multi resistant isolate that were resistant to all the four antibacterial agents (Ciprofloxacin, Ofloxacin, Pefloxacin & Sparfloxacin) were taking for curing experiment.

Plasmid Curing Experiment

Isolates which proved resistant to the FQs, were subjected to standard plasmid curing experiment. Isolates were cultivated in graded concentration of the curing agent. (Silhary *et al*., 1984) and plasmid mediated FQ-resistant isolates were subjected to plasmid extraction and characterization.

A typical experimental protocol is as follows:

- Overnight broth culture inoculated into 4.5ml nutrient broth;
- 0.5ml SDS added as the curing agent.
- Mixture incubated for 48hrs at 37^{o}C.
- 0.5ml of the broth added into a freshly prepared 4.5ml nutrient broth and incubated for another 24hrs at 37^{o}C, after which sensitivity test is carried out again.

The reason for this second susceptibility test is to check if the curing agent has removed the resident plasmid present in the resistant isolates. And if after the sensitivity test is done and some of the isolate is still resistant .It is due to chromosomal mediated efflux pump but if sensitive it is plasmid mediated, both the chromosomal and plasmid mediated clinical isolated were further taking for plasmid profiling.

Plasmid Analysis

Plasmid DNA was extracted cultured cells following alkaline lysis method of plasmid preparation, Kotchoni *et al*., 2003. The samples were processed using gel electrophoresis to identify the number of plasmid copies present in different isolates. For this purpose, an agarose gel of 0.8% was used. Staining of DNA fragments was carried out using ethidium bromide and they were visualized by UV-Trans illumination. Standard DNA molecular weight markers were used to estimate the plasmid size. The standard DNA molecular weight Markers used in the present study were, 1kb ladder, 1kb plus DNA ladder and DNA/*Mlu* I digest.

The plasmid profiling is carried out by sub-culturing those isolate onto nutrient agar plate and incubated for 24hrs at 37^{o}C, there after, the isolate is harvested into Eppendorf tube, then about 200µl of buffer 1A is added and vortex for proper mixing. Add 400µl of lysing solution. Invert the tubes for 20 times at room temperature. Add 300µl of ice-cold buffer 2B, vortex and keep once for 30 minutes, centrifuge at 2000xg for 15 minutes. To supernatant, add 700µl of chloroform vortex, centrifuge at 3000xg for 10mins. To 500µl aqueous layer, add 1ml of absolute ethanol, keep in ice for 1 hour, centrifuge at 3000xg or 30mins wash pellet $\leq$ 70% ethanol. Decant and dry pellet add 100µl of buffer C,

After the DNA have been extracted from the cell the 0.8%agarose gel is prepare for electrophoresis in which above 10µl of 10bp molecular marker mixed in loading dyes in the 1st well. Then load 10µl samples + 2µl loading Dye in other wells of agarose. Run at 90v for 60mins. View under UV Tran illumination for the size of each band harboring plasmid.

Results

The results obtained are shown in Tables 1 – 4. Table 1 shows the clinical isolate of Pseudomonas aeruginosa characterized from the different sites. Table 2 shows the susceptibility pattern of pseudomonas aeruginosa isolate to some fluoroquinolones such as Ciprofloxacin (61.1%), Ofloxacin (52.8%), Pefloxacin (56.3%) and Sparfloxacin (44.4%) at the pre-antibiogram stage.

While Table 3 shows the post-antibiogram of *Pseudomonas aeruginosa* after curing to the same fluoroquinolones above with Ciprofloxacin (5(31%), Ofloxacin (2(12%), Pefloxacin 2 (12.5%) and Sparfloxacin (3 (15%) other were still resistance to the FQs.

Table 4, also the number of clinical isolate susceptible to the four FQs at the pre-antibiogram. Figs. 1 and 2 show the bar-chart of the clinical isolate of *Pseudomonas aeruginosa* at pre and post antibiogram and while figure III show the frequencies of *Pseudomonas aeruginosa* in the sample sources.

Table 1: Isolate characterized from the various sources

Source/site	Number of isolate	Biochemical reaction/profile						
		Citrate	oxidase	Urea	Lactose	Motility	Growth 42^{o}C	Gram Reaction
Ear swab	2	+	+	+	-	+	+	-
Urine	7	+	+	+	-	+	+	-
Wound swab	20	+	+	+	-	+	+	-
Sputum	3	+	+	+	-	+	+	-
Catheter	0	+	+	+	-	+	+	-
Semen	4	+	+	+	-	+	+	-
Total	**36**							

Table 2: Pre Antibiogram of *Pseudomonas aeruginosa*

Population	**Cpx**	**Ofx**	**Pef**	**Spx**
Sensitive Resistant	22 (61.1%)			
N = 36	14 (39.0%)			
Sensitive		19 (52.8%)		
Resistant		17 (47.0%)		
N = 36				
Sensitive			20 (56.0%)	
Resistant			16 (44.4%)	
N = 36				
Sensitive				16 (44.4%)
Resistant				20 (56.0%)
N = 36				

Table 3: Post Antibiogram of *Pseudomonas aeruginosa*

Population	**Cpx**	**Ofx**	**Pef**	**Spx**
Sensitive Resistant	5 (36.0%)			
N = 14	9(64.3%)			
Sensitive		2 (12%)		
Resistant		15 (88%)		
N = 17				
Sensitive			2 (12.5%)	
Resistant			14 (87.5%)	
N = 16				
Sensitive				3 (15%)
Resistant				17 (85%)
N = 20				

Note: *N = Number of Isolate*

Table 4: Shows serial number of individual isolate susceptible to the 4 FQs at pre-antibiogram

S/N	Cip	Ofx	Pef	Spx
5	S	S	S	S
10	S	S	S	S
13	S	S	S	S
30	S	S	S	S
138	S	S	S	S
142	S	S	S	S
111	S	S	S	S

Note: S = Sensitive

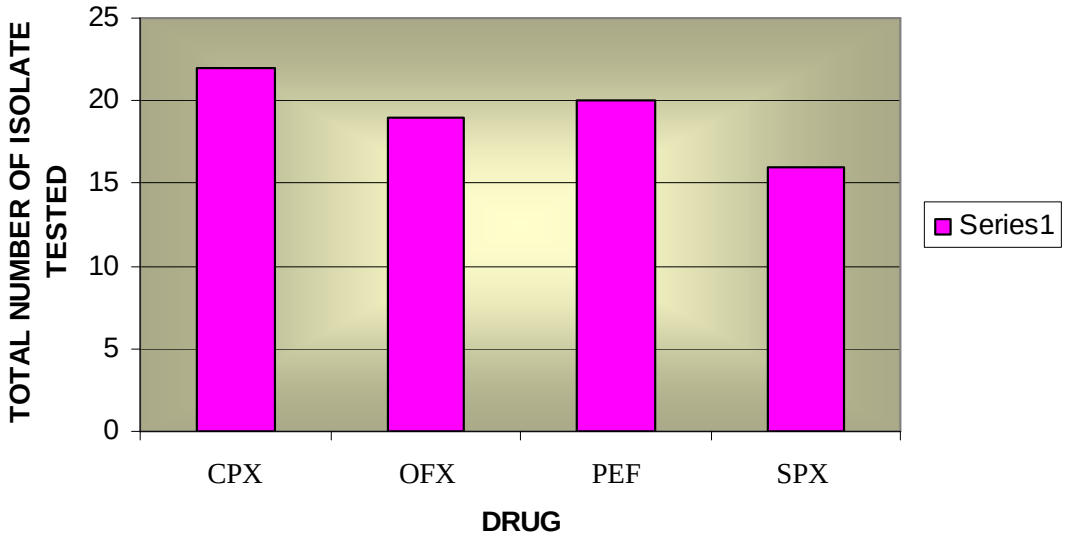

FIGURE 1: BAR CHART SHOWING THE PRECURING ANTIBIOGRAM FOR PSEUDOMONAS AERUGINOSA AMONG THE FOUR ANTINBIOTIC USED

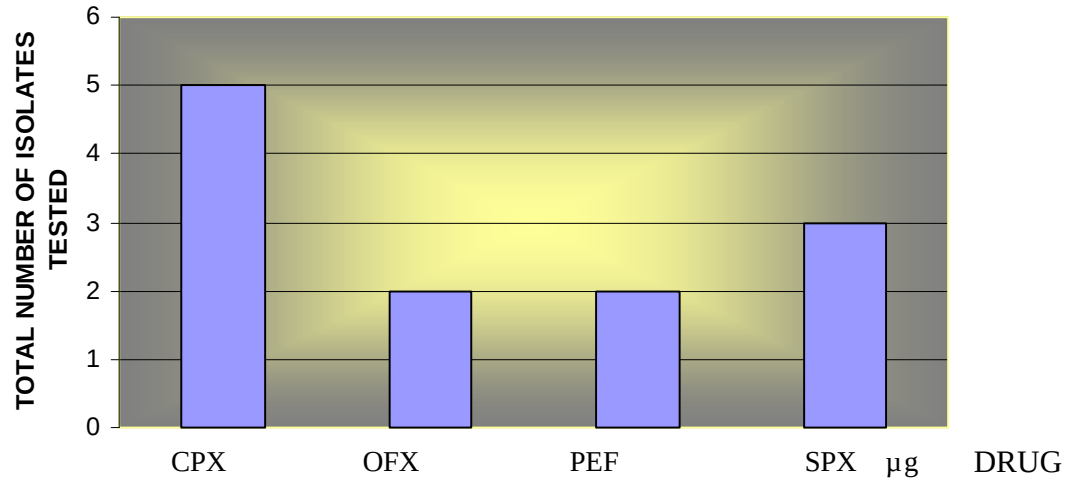

FIGURE 2: BAR CHART SHOWING POST CURRING ANHTIBIOGRAM AND MOST EFFECTIVE ANTIBIOTIC FOR PSEUDOMONAS AERUGINOSA.

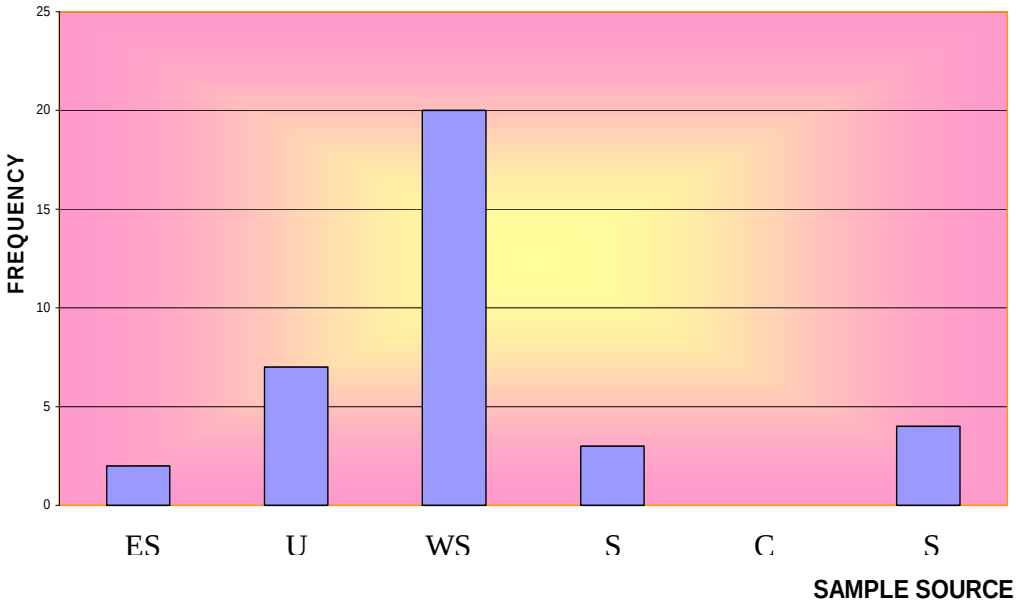

FIGURE3: BAR-CHART SHOWING THE FREQUENCY OF PSEUDOMONAS IN THE SAMPLED SOURCES.

Key:
ES= EAR SWAB, U= URINE, WS= WOUND SWAB, S=SPUTUM, C= CATHETER, S= SEMEN

Discussion

This study was performed to investigate the resistance of clinical isolates of Pseudomonas aeruginosa to some FQs due to efflux mechanism expressed by these organisms or its plasmid mediation.

Thirty six (36) species of Pseudomonas aeruginosa were isolated from clinical specimen (Ear swab, urine, wound swab, semen and catheter specimen etc), among which wound swab shows the highest Pseudomonas aeruginosa implication (See Table 1 and Fig 3i).

The susceptibility pattern of clinical isolates of *Pseudomonas aeruginosa* to ciprofloxacin, Ofloxacin, Pefloxacin and Sparfloxacin was investigated and the result shows that 39% of the organism was found to be resistant to Ciprofloxacin, 47% to Pefloxacin, 44% to Pefloxacin and 56% to Sparfloxacin. This result agrees with what Paulsen *et al*., (1999). This stated that Pseudomonas aeruginosa possesses EmrE efflux pump of the sub family of small multidrug resistance family which, play an important role in the intrinsic resistance of the organisms to FQs and other antibiotics. And this is in Table 2, ciprofloxacin was the most active against the organism followed by pefloxacin, ofloxacin and least sparfloxacin like wise after the resistant clinical isolate have been exposed to curing agent for 24hrs. Ciprofloxacin was still the most active drugs, followed by Pefloxacin with 20% Sparfloxacin 15% and Ofloxacin 12%; then other still resistant to the same FQs such as Cip (64%), Ofx (88%), Pef (87.5%) and Sparf (85%), this also agree with the work of (Chen *et al.,* 1995) that some were chromosomally mediated while others were plasmid mediated.

Table 4 shows the susceptibility pattern of some individual clinical isolate of *Pseudomonas aeruginosa* been sensitive to the four FQs used in this study at pre-antibiogram phase. This agree with the work of Rhmantis 2000 that these clinical isolates of *Pseudomonas aeruginosa* does not hyper expressed one of the efflux system such as MexAB-OprM that confer intrinsic resistance to *pseudomonas aeruginosa* and other antibiotic.

This hyper expression of MexAB-OprM efflux pump merely found in *Pseudomonas aeruginosa* was not found in any of these *Pseudomonas aeruginosa* isolate hence they are susceptible to the drug used, this is an indication that in the mist of the multidrug resistant *Pseudomonas aeruginosa* isolates, there are some isolates that are as well sensitive to the drugs used in the study.

Ciprofloxacin was also reported to be of higher effectively among the FQs tested. See table 2 and 3. This effectively was not absolute, reflecting a decline in the once considered to be susceptible among this organism to FQs. Also FQs resistance is more likely due to mutation of the target state for the various drugs or an altered DNA gyrase.

After the curing process, the post-curing antibiogram shows about 79% of resistance to FQs used, this resistance is most likely due to a stepwise chromosomal mutation of the drugs (FQ) target site or an altered DNA gyrase. This is also in accordance with Ruiz (2003) work.

The result obtain from this work also show the three components active efflux system in *Pseudomonas aeruginosa* which are responsible for multidrug resistance, these are MexA, MexC, Mex E and Mex X which are located in the periplasmic space and links Mex B – Mex A – OprM and Mex X – Mex Y – OprM efflux systems of the first and second components, participated simultaneously in intrinsic (natural/chromosomal) and acquired antimicrobial resistance common in *Pseudomonas aeruginosa* tested. This was previously reported by (Lawrence and Barret, 2004; Poole 2002; Kohler *et al.,* 1999).

It is also of pertinent to note that some of the *pseudomonas aeruginosa* tested are not exhibiting their wild type nature; the result in table iii indicates that there is high resistance in all FQs used compare to their susceptibility.

The plasmid profile of most multidrug resistance isolates were also analyzed which shows that 33% of the resistant isolates are plasmid-mediated as reported by Robicsek *et al*., (2006), Lawrence and Barret, (2004) and Zyang *et al.,* (2008). Such plasmid with size greater than 1.5kpb as shown in Table IV after plasmid DNA was electrophoresed. Significant plasmid DNA molecule size uniformity was also observed, which indicates that such plasmid mediation has a similar origin and organisms are closely related.

The overall resistance rates in this study were lowest with ciprofloxacin thus, this drug remain rational choice for empirical treatment of serious infection as well as management of hospitalized patients infected with *Pseudomonas aeruginosa*.

Conclusion

The characteristic intrinsic antimicrobial resistance of Pseudomonas aeruginosa owns much to the presence of tripartite MDR efflux system in this organism as does the acquired fluoroquinolones, efflux substrate extend well

beyond clinically relevant agents, signifying that antimicrobial efflux and resistance is not the intended function of these systems.

Recommendations

- The curing agent acts on the antibiogram and change the nature of the susceptibility of Micro-organism.
- Efflux proteins were also observed as gene preserve in the plasmid DNA (Extra-chromosomal DNA) of micro-organism as the post curing results indicates a charge since plasmid DNA has been removed.
- There is need for a further study on an invivo test to check in some patient that have micro-organism that express efflux mechanism, and how such patients would be managed in the hospital (medical environment).

References

1. Kohler T, Vandelden C, Curty L K, Ham Zehpour M M, and Pechere J C. (1997): Over expression of the Mexef-Oprn Multi-drug efflux system affects cell.
2. Henderson D K. (1988): Indolent epidemic of *Pseudomonas* in an intensive care unit traced to a contaminated blood gas analyzer. *Am. J. Med.* **84:** 75.
3. Bodey G P (1983): Infection caused by *Pseudomonas aeruginosa*. *Rev. Infec. Dis.* **5**:279.
4. Jones R J, Roe E A and Gupta J L (1980): Controlled trial of *Pseudomonas* immunoglobin and vaccine in burn patients. *Lancet.* **2:** 1263.
5. Lawrence L E and Barret J E (2004): Efflux pumps in bacteria overview, clinical relevance and potential pharmaceutical target. *Exp. Opin. Investi-drugs* **7:** 199-127.
6. Poole K. (2002): Mechanism of bacterial biocide and antibiotic resistance. *J. Appl. Microbial*. Symposium supplement. **92**:55-64.
7. Cowan S T and Steel S (1974): Manual for the identification of Medical Bacteria. 3rd edition, Cambridge University press.
8. Clinical Laboratory Standard Institute: Performance standard for Antimicrobial disk susceptibility tested: approved standard M2-A9 ed. 9, 2006 CLSi and M100-E16 NCCLS (CLSI 2006).
9. Sijhary T J, Bermann M L and Enquist L W (1984). Experiments with Gene fusions. Cold spring Habor, NY; Cold spring Habor Laboratory press.
10. Kotchoni E, Gachomo E, Betiku O O and Shonukan T (2003).Plasmid analysis using alkaline lysis. Africa Journal of Biotech. **2**(4). 88-90.
11. Paulsen I T, Brown M H and Skurray R A (1996): Proton-dependent multi-drug efflux systems, *Micro, bio. Rev.* 60: 575-608
12. Chen H Y, Yunn and LiverMore, DM, (1995): Mechanism of resistance to beta-lactam antibiotics among *Pseudomonas aeruginosa* isolates collected. *Med microbial*. **43**:300-309.
13. Rahmatic S, Yang S, Davidson A L and Zechiedrich EL. (2002): Control of the Acr AB Multi-drug efflux pump by quorum sensing regulator. *Mol. Microbiol* **43:** 677-850.
14. Ruiz J (2003): Mechanism of resistance to Quinolones: target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy.* **51**:1109-1117.
15. Robicsek A, Strahileritz J, Jacoby G A, Macielag M, Abbanat D, Bush K and Hooper D (2006). Fluoroquinolones modifying enzyme; a novel adaptation of a common amino glycoside acetyl transferase. *Nature medicine*, **12**:83-88.
16. Zhang L and Poole K (2000). Multiple antibiotic resistances in *Pseudomonas aeruginosa*: involvement of a multidrug efflux system. *Antimicrobial Agent and Chemotherapy*. **44**:287-293.