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Prevalence of Plasmids Mediated Pseudomonas aeruginosa Resistant Genes from Burn Wound Patients at the University of Benin Teaching Hospital Benin City, Nigeria

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ABSTRACT

The studies of the prevalence of *Pseudomonas aeruginosa* in burn wounds contamination in the ward were carried out. From a total of 166 *Pseudomonas aeruginosa* strains, 104 (62.7%) were obtained from freshly admitted burn wounds inmates where the isolates predominated in second-degree burns 45 (43.3%), followed by first-degree 38(36.5%) and third- degree 21(20.2%). From long term patients, a total of 62 (37.3%) were obtained where the isolates dominated in third degree burns 33(53.2%) and second- degree 29(46.8%). The organisms were further tested for their antibiotic sensitivity pattern. The quinolones, cefuroxime and gentamicin were the most effective on the isolates from newly admitted patients and to a lesser extent on isolates from long-term patients. Generally, there was a significant difference between ($p<0.05$) the antibiotic sensitivity pattern from freshly admitted patients and long hospitalized patients. The resistant plasmids to the various strains were very diverse and distributive. They were highly transferable with a frequency range of $2x10^{-2}$ to $6x10^{-4}$. Some of the strains had plasmids bands that ranged from $\leq$0.55kbp to $\geq$1.14kbp. This indicates that plasmids allow the movement of genetic materials, including antimicrobial resistance genes between bacterial strains.

INTRODUCTION

Hospitals worldwide are facing unprecedented crisis due to increasingly rapid emergence and dissemination of antimicrobial resistant *Pseudomonas aeruginosa* in burn wounds and its environs[34]. Strains of *Ps. aeruginosa* resistant to antimicrobial agents including the quinolones are endemic already in numerous hospitals and chronic burn unit institutions[1,7]. Pournaras *et al*[25] and Laura *et al*[15] found that *Ps. aeruginosa* was one of the leading causes of nosocomial infections, including pneumonia, urinary tract infections, and bacteremia. These infections can be particularly severe in cases of an impaired specific or nonspecific defense, such as that in neutropenic or cancer patients[24]. Therefore, acquired resistance to these agents constitutes a major challenge for anti-*Pseudomonas* chemotherapy, especially when it is associated with resistance to other

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classes of antibiotics, such as beta lactams, aminoglycosides and fluoroquinolones[15,16].

Patients with burns infected with *Ps. aeruginosa* suffer from a significant prolonged stay, disability, deformation, cost of the in-patient treatment and death[9,28,35]. The devastating effects are usually at large but unfortunately burn infections associated with *Ps. aeruginosa* are not yet noticeable diseases and hence no proper records or statistical data are available in most third world countries. As a matter of fact this is the greatest handicap and should be of great concern for those who treat and rehabilitate burn wounds patients. The increasing morbidity and mortality due to burn injury resulting from antibiotic resistant *Ps. aeruginosa* is on the increase therefore having a high bearing on the socio-economic status on the population[20]. The work was carried out from 2001- 2004 to determine the increasing *Ps. aeruginosa* resistance to antibiotics in relation to their plasmids profile at the University of Benin Teaching Hospital Benin City Edo State Nigeria. The information obtained, will be used to augment the present knowledge on multi antibiotics resistance in our community. This definitely, will help in developing proper measures aimed at controlling antibiotics resistance bacteria; improve the quality of antibiotics prescription and usage among burn wound patients. This will also help to trace the resistant pattern among burn wounds isolates in our environments where such trials have not been documented.

The prevalence of *Ps. aeruginosa* in burn wounds and the usual approach to the prevention of wound infection that include aseptic techniques, disinfecting procedures and well planned systemic antimicrobial therapy has failed because several strains of *Ps. aeruginosa* can now grow and degrade most of these agents[26].

Materials and Methods

A total of 104 swabs of burn wounds were obtained from freshly admitted patients and from 62 patients of the same cause who stayed for more than four months at the University of Benin Teaching Hospital (UBTH), Benin City, Edo State of Nigeria, from January 2001 to September 2004. Burns were regarded as infected when purulent drainage occurred or when the wounds fail to heal within the healing period depending on the degree of the burn[8]. All the specimens were immediately taken to the University of Benin Teaching Hospital (UBTH), Medical Microbiology Laboratory for processing.

Samples were inoculated aerobically on sterile glucose broth, nutrient agar, blood agar, MacConkey agar[6] at 37^0C for 24 hours to 48 hours. The colonies of each representative strains were then characterized using bacteriological methods[7]. Such tests included Gram stain, catalase, coagulase, oxidase, citrate test, and hemolysin production, and pigment production, growth at 42^0C, distinctive smell, and sugar fermentation. They were further sub-cultured and stored on nutrient agar slants at 4^0C for further analysis.

Antibiotic Susceptibility

The antibiotic susceptibility of freshly admitted and prolong hospitalized patients isolates were determined both by overnight broth-micro-dilution and agar diffusion as recommended by National Committee for Clinical Laboratory Standard (NCCLS)[19] using Oxoid- Mueller Hinton agar (Difco Laboratories, Detroit, Mich.). The antimi-crobial agents used included; erythromycin 10µg, cefuroxime 30µg, streptomycin 10µg, ofloxacin 5µg, pefloxacin 10µg, ampicillin 10µg, tetracycline 10µg, cloxacillin 10µg and gentamicin 10µg. Zones of inhibitions and MIC were then measured and the results recorded as sensitive (S) or resistant (R) based on World Health Organization drug Infor-mation[10] and National Committee of Clinical Laboratory Standards (NCCLS)[19].

Conjugation and Plasmids profiles

Conjugation was carried on the isolates; according to Wang *et al*[31] and Yutaka *et al*[36] with Ps. aeruginosa strain ATCC 10145 used as the recipient mating strains were incubated at 37°C for 18 hours without shaking. The transconjugants were selected on Mueller Hinton agar medium supplemented with the above antibiotics and rifampicin 100µg (Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan) to inhibit the growth of the donor and recipient respectively. The transconjugants were re-streaked onto fresh Mueller Hinton agar plates and their identities were re-confirmed on the basis of their biochemical methods, pigment production and their antibiotics resistance pattern. The frequency of transfer was determined by dividing the number of transconjugants by the number of donor cells. The Birnboim and Doly[5] method was employed for screening of plasmids (rapid alkaline extraction) of donors and transconjugants.

The plasmids DNA were then electrophoresed on 0.8% agarose gel, stained with 14µl ethidium bromide. The DNA was then photographed and viewed using UV transillumination. The molecular weights and distances moved were then determined using standard methods according to Meyers *et al*[17] and Birnboim and Doly[5]. The bacteriophage lambda HindIII (Roche Diagnostic GmbH) was included in each extraction experiment as control and as standard molecular weight marker. Transconjugants and cured bacterial cells were similarly treated.

Curing Experiments

Curing of isolates was carried out using the modifications of the Olukoya & Oni[22] and Miller[18] methods. Overnight cultures in nutrient broth were diluted 10-fold and 1ml inocula were added to 30ml of nutrient broth (pH of 7.6). The cultures were then incubated for 24 hours with 1ml 10% of sodium dedocyl sulfate (SDS) solution. The overnight broth cultures were diluted with sterile distilled water and cultured on Mueller Hinton agar plates. The colonies were then sub-cultured onto Mueller Hinton agar (Difco Laboratories, Detroit, Mich) plates to test run for their respective antibiotic sensitivity patterns as previously described. Resistance markers expressed after curing were regarded as being chromosome-mediated while those that were not expressed were regarded as plasmid-mediated.

Result

The *Ps. aeruginosa* strains isolated from burn wounds on freshly admitted, prolong hospitalized patients are as shown in Table (1). From freshly admitted patients, the bacterial strains predominated in second-degree burns 45(43.3%), followed by first degree 38 (36.5%) and third degree 21(20.2%). From prolong hospitalized patients; the prevalence was high in third degree 33(53.2%), followed by second degree 29(46.8%) and no first-degree sample because the patients were all treated. The antibiotic (Oxoid multi-discs) susceptibility patterns are

Table 1: **Prevalence of *Ps. aeruginosa* from various degrees of burn wounds; from freshly admitted and from long-term patients after 16 weeks of hospitalization**

		Degree of Burn		
Samples	**No. of Isolates**	**1°**	**2°**	**3°**
Freshly admitted patients	104(62.7%)	38 (36.5%)	45 (43.3%)	21(20.2%)
Long Term patients	62(37.3%)	_	29(46.8%)	33(53.2%)
Total	166 (100%)	38 (22.9%)	74 (44.6%)	54 (32.5%)

KEY: 1° = First Degree Burns, 2° = Second Degree Burns, 3° = Third Degree Burns

as shown in Table 2, where the strains were more sensitive to the quinolones, followed by the aminoglycosides and the ampicillin the least. The resistance plasmids to the various isolates were very diverse and distributive among the isolates as shown in Table 3. They were also highly transferable with a frequency range of $2x10^{-2}$ to $6x10^{-4}$. Some of the isolates had plasmids bands that ranged from $\geq$0.55kbp to $\leq$1.14kbp. 11.4% of the isolates were plasmids mediated, 48.9% chromosome mediated while 39.7% antibiotics resistance of the isolates could not be ascertained. Longed hospitalization of patients resulted into high emergence of resistant strains of *Ps. aeruginosa*.

Table 2: **Antibiotic susceptibility pattern of *Ps. aeruginosa* from freshly admitted and from long-term patients.**

Source of samples	No of isolates	OB	CN	PN	TE	E	S	CXM	OFX	CIP	PEF
Fresh Admission	104	11.5%	58.7%	5.8%	34.6%	50%	47.1%	55.8%	67.3%	86.5%	76.9%
Prolong Admission	62	0.0%	37.1%	0.0%	24.2%	22.2%	25.8%	19.4%	54.8%	51.6%	46.7%

KEY: OB = Cloxacillin, CN = Gentamicin, OFX = Ofloxacin, PN = Ampicillin, TE = Tetracycline, CIP = Ciprofloxacin, E = Erythromycin, PET = Pefloxacin, CXM = Cefuroxime S = Streptomycin

Table 3: **Antibiotic Resistance and Plasmid Profile of Ps aeruginosa Isolates Obtained from Burns patients wounds from University of Benin Teaching Hospital (UBTH) from 2001to 2004**

Isolates Code	Antibiotics	Plasmids size (kbp) donor	Transferred Plasmids	Frequency Size (kbp)	Resistant marker after of Transfer
01	OB,CIP,S,CN,TE,PN,E	1.14, 0.85, 0.55	1.14, 0.85	$6x10^{-4}$-	S,CN,TE
1	CIP,PN,CN,	1.08, 0.79	1.08, 0.79	$3x10^{-4}$	CN, PN
2	CIP,CN	-	-	-	-
3	PEF,CIP,S,PN	1.14, 0.85	1.14	$2x10^{-2}$	PN,S
5	CN,S,OB,CXM,PN	1.14, 0.85	1.14,	$6x10^{-2}$	CN,S
6	TE,CN,PEF,CXM,S	1.08, 0.79	1.08, 0.79	$3x10^{-1}$	CN,S
9	CIP,CN,TE	-	-	-	-
15	CN,CIP,E,PN,S	1.14	1.14	$6x10^{-2}$	E,S,CIP
20	CN,OFX,TE,S,E	1.14, 0.85	0.87	$6x10^{-2}$	S,E
40	TE, PN, CIP,PN,CXM	1.08	1.08	$2x10^{-2}$	TE,PN
18	PN, CN,S,E,CXM	1.08, 0.79	1.08	$3x10^{-4}$	PN,CN,S
56	CXM, TE,S,PN,CN	1.08, 0.79, 0.55	0.79, 0.55	$6x10^{-2}$	CN,PN,TE
21	TE, S,CN,PN,CXM,E	1.14, 1.08, 0.85	1.14, 0.85	$6x10^{-2}$	TE,S,CN
48	S,PN,CIP,CXM,TE	1.08	1.08	$6x10^{-2}$	TE,S
62	CN,OFX,TE,S,E	1.14, 0.85	0.87	$6x10^{-2}$	TE,S,E
37	CN,OFX,TE,S,E	1.14, 0.85	0.87	$6x10^{-2}$	TE,S,E
99	CN,OFX,TE,S,E	1.14, 0.85	0.87	$6x10^{-2}$	TE,S,E
101	CN,OFX,TE,S,E	1.14, 0.85	0.87	$6x10^{-2}$	TE,S,E
102	CN,OFX,TE,S,E	1.14, 0.85	0.87	$6x10^{-2}$	CN,TE,S

KEYS: OB = Cloxacillin, CN = Gentamicin, OFX = Ofloxacin, PN = Ampicillin, TE = Tetracycline, CIP = Ciprofloxacin, E = Erythromycin, PET = Pefloxacin, CXM = Cefuroxime, S = Streptomycin

Discussion

The results indicated that *Ps. aeruginosa* could be implicated in various degrees of burn wounds as shown in the present study and other literatures[20,4,28]. The *Ps. aeruginosa* strains implicated in freshly hospitalized patients are as shown in Table 1 where *Ps. aeruginosa* predominated, as reported in the findings of[20]. This is due to the fact that *Ps. aeruginosa* is an ubiquitous isolate; hospital base opportunistic organism hence can easily gain access into the tissues when the integrity of skin is destroyed[27,26]. Damage to the skin exposes the tissues to organisms of low virulence due to the impairment of the host immune responses[8,27].

According to the studies of Pittet *et al*[23], Vindene *et al*[30], Stevens *et al*[29] and Yah *et al*[34], hospital staff are carriers of *Ps. aeruginosa* as well as on their clothing, bed linens and other formites in human environment[26]. In this case they can easily shed these *Ps. aeruginosa* strains to these debilitated wounds. This correlated with the studies of Pittet *et al*[23] where they found that *Ps. aeruginosa* was the leading nosocomial pathogen in Swiss University hospitals and highly virulent to immunosuppressed patients. Also when Nagesha *et al*[20] subjected various degrees of burns to bacteriological and clinical examination the commonest organisms on admission were *Ps. aeruginosa* and Staph. aureus and were of very high prevalence on deep burn wounds. *Ps. aeruginosa* is usually found in sinks, water bath, bathtub and water taps[13,3,34,35] as a result could easily be transmitted to patients when they are on the facilities. Similar findings had also showed that the abundant and extensive/abusive use of antimicrobial agents particularly in hospitalized patients had led to the suppression of drug susceptible organisms in burn wounds therefore favouring the persistent growth of drug resistant bacteria. Also the close environment of the hospitals had also favoured the transmission of such resistant organisms through formites as well as direct contact[14,12,32] and from dressing rooms.

Additional institutional factors that may increase the likelihood of person to person transmission include resident interaction in two or four-bed rooms and communal activities such as meals and various types of therapy, high patient-to- staff ratios facilitate cross infection[11]. Findings have also suggested that person to person spread is through direct contact especially that between a resident and the transient colonial hands of a health care worker, is thought to be the principal mode of transmission[33]. Isolation of resistant pathogens from hands of health care workers and observations on timing of new cases have provided some evidence for methicillin resistance *Staphylococcus aureus* (MRSA), resistant gram-negative *Ps. aeruginosa* and *uropathogens E. coli*[33,14].

One of the essential features in the infectious process is the colonization and invasion of burns by *Ps. aeruginosa* with the elaborate production of virulence enzymes such as elastase, hyaluronidase, collagenase, and lipases[9,26,3] that enhance the infectious process. This could highly influence the rate of morbidity and mortality in severe burn patients on longed hospitalization.

As seen in Table 2, there was a change in the antibiotic susceptibility patterns of the isolates from freshly admitted to long-term patients. The antibiotics that were effective on isolates from freshly admitted became ineffective on isolates from longed hospitalization. The result indicated that the patients were able to acquire resistance to *Ps. aeruginosa* when they were on the facility[14]. These antibiotic susceptibility disparities are shown in Table 2 although the quinolones (CIP, OFX and PET) showed some effectiveness, this was subject to the fact that they are new generation antibiotics, expensive and have not been exposed to extensive abuse in our community.

The prolonged hospitalization of patients resulted in high emergence of resistant strains

of *Ps. aeruginosa*. It is now hoped that with increasing resistance to antimicrobial agents, antibiotics chemotherapy may be replaced by immunization of those at risk against typical *Ps. aeruginosa* burn wound infections. The study also demonstrated that Ps. aeruginosa isolates from burn wound samples in UBTH harbored multi drug resistance conjugative plasmids, which may confer resistance to some of these other antibiotics. They were also highly transferable with a frequency range of $2x10^{-2}$ to $6x10^{-4}$. Some of the isolates had plasmids bands that ranged from $\leq$0.55kbp to $\geq$1.14kbp. 11.4% of the isolates were plasmids mediated, 48.9% chromosome mediated while 39.7% antibiotics resistance of the isolates could not be ascertained. The number of plasmid bands per isolate, did not reflect the nature of resistant markers. The plasmids molecular weights obtain in this work were very small as compare to plasmids isolated by other literatures with very large molecular weight plasmids[9,2,22]. According to Aluyi and Akortha[2], these multiple copies of plasmid bands might have resulted from covalently close circular, open circular and linear forms of the same plasmid that migrated at different rates on agarose gel electrophoresis.

Most of the antibiotics resistance plasmids could have been lost due to successive sub-cultures[26] that may have been the reasons why 39.7% resistance could not be ascertained. Nashwan *et al*[21] described that antibiotic resistance genes are often carried in mobile genetic elements and the transfer of these genes between different bacterial species may go unnoticed by traditional infection control and epidemiological methods, thereby undermining hospital infection control policies. Traditionally, the role of antibiotics in antibiotic- induced antimicrobial resistance is to provide selective pressures to resistant clones. More to those stringent measures directed against all multi-drug resistance especially the use of gloves and wearing of gowns, extra attention to hospital hygiene and proper hand washing can reduce plasmid antibiotics mediated resistance among isolates.

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