

Original Research Article

Plasmid Profile of Multi-Drug Resistant *Proteus* spp Isolated from Patients with Wound Infection in Northeastern Nigeria

ABSTRACT

Aims: We analyzed the prevalence of plasmid mediated multidrug resistance among *Proteus* spp isolated from wound infection patients attending healthcare centers in Maiduguri.

Methodology: 320 wound swab samples were collected and investigated via microscopy, cultured on Blood agar and MacConkey agar. Suspect *Proteus* spp isolates were further confirmed using biochemical tests. Kirby bauer disc diffusion test was used to determine the antimicrobial susceptibility pattern. Isolates confirmed to be multidrug resistant (MDR) were subjected to gel electrophoresis for the determination of plasmid profile.

Results: Twenty eight (28) samples yielded *Proteus* spp, giving a prevalence rate of 8.75%. *Proteus mirabilis* was the most significant specie isolated (32.14%). Isolates were most sensitive/least resistant to Ciprofloxacin (85.71%/14.29%) and most resistant/least sensitive to Augmentin (10.71%/89.29%). 64.28% of isolates observed were MDR strains and were quite significant among patients between the ages of 21-30years (21.43%). MDR *Proteus mirabilis* was most the significant and highly prevalent among patients suffering from wound sepsis and burns (10.71% respectively). The association between MDR *Proteus* spp and wound types was statistically not significant ($X^2 = 7.342$, $p > 0.01$). Plasmid profile analysis revealed that 72.22% of the MDR isolates harbor plasmids with a DNA fragment size of 100 bp and a molecular weight of 31ng/10 μ l. An average of 7.22% of MDR isolates were cured of their plasmids while an average of 56.67% of MDR isolates resisted curing.

Conclusion: Here, we report a high prevalence of multidrug resistance and a high rate of plasmid carrying strains of *Proteus* spp in wounds of hospitalized patients. We suggest that there is a role played by plasmid in the mediation of multidrug resistance among the MDR *Proteus* spp isolated, where the majority of the MDR isolates observed carry plasmids of similar size and molecular weight.

Keywords: *Proteus* spp, Plasmid profile, multi-drug resistance, wound infections, Plasmid curing.

1. INTRODUCTION

Wound infection can be regarded as the invasion and proliferation by one or multiple species of microorganisms on wound, which could lead to pus formation (Calvin, 1998). Wounds can be classified as accidental, pathological or post-operative. Irrespective of the nature of the wound, infection is the attachment of microorganisms to host cells. They proliferate, colonize and become better placed to cause damage to the host tissues (Collier, 2003). Wounds can be infected by a variety of bacteria, the most common of which are *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp and *Proteus* spp (Gus Gonzalez et al., 2006).

Proteus spp is a rod-shaped pleomorphic, motile, non-capsulated, aerobic/facultatively anaerobic, gram-negative bacterium that has been implicated in hospital and community acquired infections. They are widespread in the environment and make up part of the normal flora of the human gastrointestinal tract. Although *Escherichia coli* accounts for the largest percentage of cases of uncomplicated cystitis, pyelonephritis, and prostatitis, *Proteus* spp ranks third as the cause of these infections, particularly in the nosocomial setting (Stamm, 1999). Owing to their varied habitats, the modes of transmission of *Proteus* spp include nosocomial sources such as hospital food and equipment, intravenous solutions and human contact through contaminated skin surfaces. Long-term indwelling catheters are a prime site of colonization and infection for *P. mirabilis* and *P. vulgaris* (Stickler and Hughes, 1999).

Proteus spp have been implicated in the infection of wounds especially diabetic wounds along with *Escherichia coli*, *Enterobacter* spp, *Klebsiella* spp and *Serratia marcescens* (Goldstein et al., 1996;

Mutta *et al.*, 2003; Ravisekhar *et al.*, 2006). They cause significant clinical infections which are difficult to eradicate, especially from hosts with complicated wounds, catheterization and underlying diseases as well as the immunocompromised (Auwaerter, 2008). *Proteus* spp can be naturally resistant to antibiotics; some strains produce penicillinase and almost all are resistant to the polymyxins such as colistin and polymyxin B (Duguid *et al.*, 1987), but the most disturbing aspect was the emergence of multi-drug resistant strains that are spreading incessantly (Armbruster and Mobley, 2012).

The prevalence of plasmid mediated multi-drug resistant (MDR) bacterial isolates has increased in the last few years, affecting the prognosis and survival of hospitalized patients especially in developing countries like Nigeria. Like many other members of the family *Enterobacteriaceae*, *Proteus* spp can harbor numerous plasmids and integrons that houses the genetic determinants of antimicrobial resistance (Hall and Collis, 1998). Multidrug-resistant (MDR) strains of *P. mirabilis*, for instance, produce extended-spectrum β -lactamases (ESBLs) or the AmpC-type cephalosporinase and rarely carbapenemases, and their prevalence in some settings is relatively high (Cohen-Nahum *et al.*, 2010; D'Andrea *et al.*, 2011).

We investigated a cohort of hospitalized patients suffering from wound infection to ascertain the prevalence of multidrug resistant *Proteus* spp from the various wound types, and to look at the plasmid profile of the isolates which shall indicate the implication or otherwise of plasmid as a possible vector of multidrug resistance genes among *Proteus* spp.

2. MATERIAL AND METHODS

2.1 Study area

The study was conducted in Maiduguri, located in the northeastern part of Nigeria which lies within latitude 11.15 °N and longitude 30.05 °E in the sudano-sahelian savanna zone, with a dense population that are mostly crop farmers, fishermen, herdsman and traders (Udo, 1978).

2.2 Study population

The target population for the study includes in-patients and out-patients attending four healthcare centers within Maiduguri metropolis. These centers serve a population of over 20 million in the North-eastern sub-region of Nigeria, comprising six states (Borno, Bauchi, Yobe, Adamawa, Taraba and Gombe) as well as a sizeable number across the borders of Cameroon, Chad and Niger Republic (Umar *et al.*, 2016).

2.3 Sample collection and processing

Swab sample of wounds were collected at random from consented patients in both inpatient and outpatient departments. Wound beds were prepared before sample collection using Levine's technique as described by Gardner *et al.* (2007). The wound surface exudates was cleaned off with moistened sterile gauze and sterile normal saline solution. Aseptically, the swab stick was rotated over 1cm² area for 5 seconds with sufficient pressure to express fluid and bacteria to the surface from within the wound tissue. The wound swab samples were transported in 0.5ml sterile normal saline solution for microbiological analysis.

2.4 Microbiological Analysis

The wound swab samples were cultured onto 5% Blood agar and MacConkey agar plates and incubated aerobically at 37°C for 18-24 hour. Suspect colonies were further subcultured to obtain pure culture. Gram stain was conducted as a preliminary test. Pure culture of *Proteus* spp was isolated and identified based on morphological appearance, motility, Gram stain reaction and biochemical tests which include; phenylalanine deaminase, urease, hydrogen sulphite production, indole, methyl red, voges proskauer, citrate, maltose fermentation and ornithine decarboxylase test (Baker *et al.*, 2007; Cheesbrough, 2006).

2.5 Antimicrobial Susceptibility Test

2.5.1 Preparation of bacterial inoculum

Isolates of *Proteus* spp were inoculated into Nutrient broth and incubated at 37°C for 5 hours until turbidity was adjusted to 0.5 Mcfarland turbidity scale. This turbidity scale was prepared by adding 9.6 ml of 1% aqueous solution of barium chloride (BaCl₂) in 0.4ml of 1% sulphuric acid (H₂SO₄) giving an approximate bacterial density of 1.2x10⁹ cfu/ml (Cheesbrough, 2006).

84 **2.5.2 Sensitivity Testing of *Proteus* spp Isolates**

85 The Kirby-Bauer disc diffusion method as described by Akubuenyi *et al.* (2011) was used for this test.
86 Pure isolate of *Proteus* were tested against selected antibiotics using Gram negative multidisc
87 cartridge containing the following antibiotics; Ceporex (10 µg/ml), Gentamicin (10µg/ml), Augumentin
88 (30µg/ml), Nalidixic acid (30µg/ml), Streptomycin (30 µg/ml), Ofloxacin (30 µg/ml), Pefloxacin (10
89 µg/ml), Ciprofloxacin (10 µg/ml), Ampicillin (30 µg/ml) and Cotrimoxazole (30 µg/ml).

90 The inoculum prepared (1.2×10^9 cfu/ml) was seeded onto prepared Mueller Hinton agar (MHA) under
91 aseptic conditions and the surface is allowed to absorb. The antibiotic-impregnated multidisc was
92 carefully placed onto the surface of the seeded plate with the aid of sterile forceps and incubated at
93 37°C for 18-24 hour. The zones of inhibition were then measured and interpreted according to the
94 Standard Performance Chart by Clinical Laboratory Standards Institute (CLSI, 2016).

95 **2.6 Plasmid Curing Analysis**

96 Multidrug resistant isolates were subjected to plasmid curing using the method of Isibor and Uwabor,
97 (2015). Overnight cultures in nutrient broth were diluted 10-fold and 1ml inoculum was added to 30ml
98 of nutrient broth (pH=7.6). 1ml of 10%w/v Sodium Dedocyl Sulfate (SDS) solution was added to the
99 broth and incubated for 24 hours. The overnight broth cultures were diluted with sterile distilled water
100 and inoculated onto Mueller Hinton agar plates. The colonies were then sub-cultured onto Mueller
101 Hinton agar plates and were again screened for antibiotic resistance by the disk diffusion method.
102 Resistance markers expressed after curing were regarded as being chromosome-mediated while
103 those not expressed were regarded as plasmid mediated.

104 **2.7 Plasmid DNA Extraction and Gel electrophoresis**

105 Plasmid DNA extraction was performed using the alkaline lysis method described by He, (2011).
106 After isolation of plasmid DNA, a horizontal agarose gel electrophoresis was carried out according to
107 the method of Meyers *et al.*, (1996). The DNA bands were interpreted according to the Solis BioDyne
108 DNA Ladder molecular weight marker interpretation chart (Promega Corporation).

109 **2.8 Data Analysis**

110 Data generated were analyzed using Statistical Package for Social Sciences (SPSS, version 16.0).
111 Data were presented as frequencies and percentages. Chi-square was used and evaluations were
112 carried out at 99% confidence level and $P < 0.01$ was considered as statistically significant.

113 **3. RESULTS AND DISCUSSION**

114 **3.1 Prevalence of *Proteus* spp in relation to patients' demographic properties**

115 Twenty eight (28) out of the three hundred and twenty (320) samples analyzed yielded *Proteus* spp,
116 giving a prevalence rate of 8.75%, which was more significant among males (60.71%) than females
117 (32.29%). Rate of infection was highest among patients of 21-30years (28.57%). Male patients
118 between the age categories of 21-30years recorded the most significant infection rate (21.43%) on
119 sex versus age group basis (Table 1). Similar findings by Torpy *et al.* (2005) was reported and they
120 assert that age significantly affects the prevalence of wound infections, since adolescents and active-
121 age adults are usually the ones involved in much physical activities such as sports and farming which
122 may expose them more to injuries and infections. Infants and older age individuals are also
123 considered at risk of acquiring wound infections because of the decrease in immune competence with
124 age (Omole and Stephen, 2014; Hakim and Gress, 2007), and infants have their immune system at
125 the developmental stage.

126 **Table 1. Distribution of *Proteus* spp according to patients' demographic properties**

Age group (Years)	Sex of patients		Total (%)
	Male (%)	Female (%)	
0-10	2 (7.14)	1 (3.57)	3 (10.71)
11-20	3 (10.71)	2 (7.14)	5 (17.86)
21-30	6 (21.43)	2 (7.14)	8 (28.57)
31-40	3 (10.71)	2 (7.14)	5 (17.86)

41-50	1 (3.57)	2 (7.14)	3 (10.71)
51-60	1 (3.57)	1 (3.57)	2 (7.14)
61-70	0 (0.00)	1 (3.57)	1 (3.57)
>70	1 (3.57)	0 (0.00)	1 (3.57)
Total (%)	17 (60.71)	11 (39.29)	28 (100)

* $\chi^2 = 3.041$; $df=7$; $p<0.01$

3.2 Antimicrobial susceptibility pattern of *Proteus* spp isolated

Antimicrobial susceptibility pattern of *Proteus* spp isolated showed that the highest sensitivity/least resistance was shown towards Ciprofloxacin (85.71%/14.29%) while the least sensitivity/highest resistance was against Augmentin (10.71%/89.29%) (Table 2). Cumulatively, Isolates were most sensitive/least resistant to Quinolones (with the exception of Nalidixic acid against whom a marked resistance was observed) and Aminoglycosides class of drugs, with an average sensitivity/resistance rate of 73.81%/26.19% and 71.43%/28.57% respectively. Resistance by isolates was highly pronounced against β -lactam antibiotics with an average resistance/sensitivity rate of 79.76%/20.24%.

Our results demonstrated a marked resistance by *Proteus* spp towards Nalidixic acid. It has been reported that the emergence and prevalence of Nalidixic acid-resistant bacteria have been more problematic in hospitals than in any other environments (Soto *et al.*, 2003). Poor adherence to recommended dosage by patients and widespread, indiscriminate use of antibiotics, which are freely available and accessible with high potential for abuse, is one of the reasons why drug resistance is on the rise.

Although variation in resistance exist between *Proteus* isolates examined, the overall most effective antibiotics were the Flouroquinolones and Aminoglycosides. The low level of resistance to these antibiotics may be due to their better efficacy and to certain extent, high price. This makes them not readily available and affordable and by extension, lower the chances of abuse. As such, the restriction of over-the-counter prescription of antibiotics and general awareness about the higher possibility of resistance by bacterial species to these drugs can help reduce the level of abuse in the society.

Table 2. Antimicrobial susceptibility pattern of *Proteus* spp isolated

Antimicrobial Agents	Concentration (µg)	Susceptibility Pattern	
		Sensitive (%)	Resistant (%)
<u>Quinolones</u>			
Ciprofloxacin (CPX)	10	85.71	14.29
Ofloxacin (OFX)	30	71.43	28.57
Pefloxacin (PEF)	10	64.29	35.71
Nalidixic Acid (NA)	30	14.29	85.71
<u>β-Lactam</u>			
Ampicillin (PN)	30	25.00	75.00
Ceporex (CEP)	10	25.00	75.00
Augmentin (AU)	30	10.71	89.29
<u>Aminoglycosides</u>			
Gentamicin (CN)	10	64.29	35.71
Streptomycin (S)	30	78.57	21.43
<u>Folate Antagonist</u>			
Cotrimoxazole (SXT)	30	60.71	39.29

64.27% of the isolates were multidrug resistant. Patients within the age category of 21-30 years recorded the highest yield of 21.43% and the least was observed among patients within the age category of 0-10yrs (0.00%) (Table 3). This is in concurrence with the findings of Feglo *et al.* (2010) in a study carried out in Ghana, and that of Nita *et al.* (2014) in India.

The latter concluded that resistance to antibiotics is an increasingly common problem and its management is a subject of concern. And that species identification and routine study of the

epidemiology of antimicrobial resistance will assist in the management and control of infections, and in therapeutic management of patients by shifting the mode of prescription of antibiotics from broad spectrum to narrow spectrum.

The evolution and spread of multi-drug resistant *Proteus mirabilis* clone with chromosomal AmpC-type beta-lactamase has been reported in Europe. Resistance to high levels of antibiotics has been ascribed in most instances to the presence of plasmids (Daini *et al.*, 2006; D'Andrea *et al.*, 2011).

Table 3. Percentage occurrence of MDR *Proteus* spp according to patients' age

Age Group (Years)	Multidrug resistant <i>Proteus</i> spp Isolated (%)
0-10	0 (0.00)
11-20	2 (7.14)
21-30	6 (21.43)
31-40	3 (10.71)
41-50	3 (10.71)
51-60	2 (7.14)
61-70	1 (3.57)
>70	1 (3.57)
Total (%)	18 (64.29)

Percentage rate of occurrence of multi drug resistance among *Proteus* spp showed that *Proteus mirabilis* (32.14%) recorded the most multidrug resistant (MDR) strains while *Proteus penneri* recorded the least (3.57%) (Table 4).

Table 4. Percentage frequency of multidrug resistance among *Proteus* spp

<i>Proteus</i> spp	Wild strains (%)	Multidrug resistant strains (%)
<i>P. mirabilis</i>	15 (53.57)	9 (32.14)
<i>P. vulgaris</i>	11 (39.29)	8 (28.57)
<i>P. penneri</i>	2 (7.14)	1 (3.57)
Total (%)	28 (100)	18 (64.29)

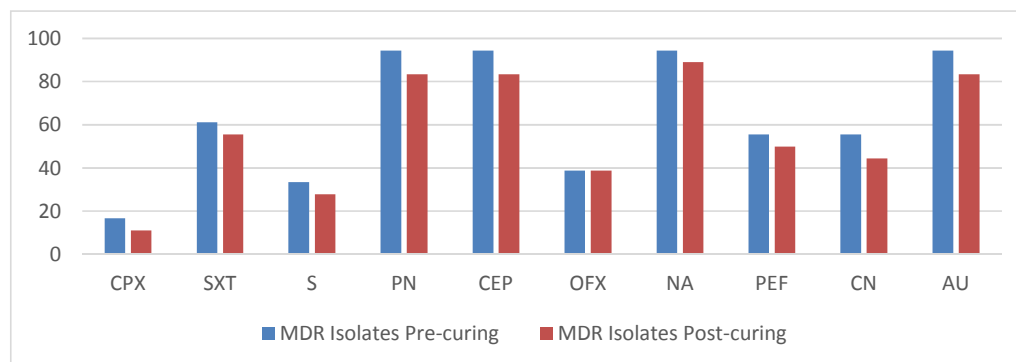
Distribution of MDR *Proteus* spp isolated from wound types examined showed that Wound sepsis and Burns wound recorded the highest prevalence (17.86% respectively), while Gunshot wound recorded the least (0.00%). MDR *Proteus mirabilis* was isolated the most from Wound sepsis and Burns wound (10.71% respectively) whereas MDR *Proteus vulgaris* was isolated the most from Wound sepsis, Wound ulcer and Burns wound (7.14% respectively). MDR *Proteus penneri* was only isolated from wound ulcer (3.57%). The association between MDR *Proteus* spp and the various wound types examined was statistically not significant ($X^2 = 7.342$, $p < 0.01$) (Table 5).

Table 5. Frequency of Isolation of MDR *Proteus* Spp among Various Wound Types Examined in the Study Area.

Wound Type Examined	Specie Type (%) (n=28)			Total (%)
	<i>P. mirabilis</i>	<i>P. vulgaris</i>	<i>P. penneri</i>	
Wound Sepsis	3 (10.71)	2 (7.14)	0 (0.00)	5 (17.86)
Wound Ulcer	0 (0.00)	2 (7.14)	1 (3.57)	3 (10.71)
Diabetic Ulcer	2 (7.14)	1 (3.57)	0 (0.00)	3 (10.71)
Post-Operative Wound	1 (3.57)	1 (3.57)	0 (0.00)	2 (7.14)
Burns Wound	3 (10.71)	2 (7.14)	0 (0.00)	5 (17.86)
Gunshot Wound	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total (%)	9 (32.14)	8 (28.57)	1 (3.57)	18 (64.29)

Plasmid curing on the 18 MDR isolates revealed an average of 7.22% of isolates were cured of their plasmids while an average of 56.67% of MDR isolates resisted curing (Figure 1). Plasmid profile analysis revealed that 72.22% of the multidrug resistant isolates harbour similar plasmids with a molecular weight of 31ng/10µl and DNA fragment size of 100bp (Figure 2). Resistance to high levels of antibiotics has been ascribed in most instances to the presence of plasmids (Daini *et al.*, 2006).

A study conducted by Yah *et al.*, (2007) reported that plasmids were able to move genetic antibiotic resistant determinants among various bacterial strains and contribute to overall pathogenic potential of disease causing bacteria. A principal mechanism for the spread of antibiotic resistance is by horizontal transfer of genetic material through conjugation, transformation, or transduction. Resistance genes can be further incorporated into the recipient chromosome (in the form of integrons) by recombination, these genes may contain single mutations or more severe sequence changes (Dzidic *et al.*, 2008). Shin-hee-kim *et al.* (2005) were able to demonstrate that plasmids containing class 1 or 2 integrons and bla_{TEM-1} were able to be transferred from *Proteus mirabilis* isolates into *Escherichia coli* by conjugation, indicating that conjugal transfer could contribute to the dissemination of antibiotic resistance genes between the *Enterobacteriaceae* species.



*CPX=Ciprofloxacin, SXT=Cotrimoxazole, S=Streptomycin, PN=Ampicillin, CEP=Ceporex, OFX=Ofloxacin, NA=Nalidixic Acid, PEF=Pefloxacin, CN=Gentamicin and AU=Augmentin.

Figure 1: Plasmid Curing Analysis of Multidrug Resistant *Proteus* Strains Isolated from the Study Area.



Figure 2: The agarose gel electrophoresis profile of plasmid DNA from *Proteus* spp isolated

4. CONCLUSION

Here, we report a high prevalence of multidrug resistance and a high rate of plasmid carrying strains of MDR *Proteus* spp in wounds of hospitalized patients. We suggest that there is a role played by plasmid in the mediation of multidrug resistance among MDR *Proteus* spp isolated, where the majority of the MDR isolates observed in this study carry similar plasmids.

206 CONSENT

207 All authors declare that informed consent was obtained from the patients (or other approved parties)
208 for sample and data collection.

209 ETHICAL APPROVAL

210 All authors hereby declare that all experiments have been examined and approved by the ethics
211 committee of the University of Maiduguri Teaching Hospital, Nigeria.

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