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Research Article
Assessment of Biofilm Forming Capability and Antibiotic Resistance in *Proteus mirabilis* Colonizing Indwelling Catheter

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Abstract
Background and Objective: Urinary tract infections from the use of an indwelling urinary catheter are one of the most common infections caused by *Proteus mirabilis*. Due to their biofilm-producing capacity and the increasing antimicrobial resistance in this microorganism, this study aimed to determine the prevalence, biofilm-producing capacity, antimicrobial resistance patterns, multidrug resistance and plasmid mediated resistance of the recovered isolates. **Materials and Methods:** A total of 50 urinary samples were collected from May to August, 2018 from patients on indwelling urinary catheters. Using routine microbiological and biochemical methods, 37 *P. mirabilis* were isolated. Biofilm forming capability was determined among the isolates using the tube method while antimicrobial susceptibility and plasmid curing were also performed. **Results:** All isolates were biofilm producers with 17(46%) being moderate producers while 20(54%) were strong biofilm formers. The study isolates exhibited a high resistance rate to empiric antibiotics, including ceftazidime (75.8%), cefuroxime (54.5%), ampicillin (69.7%) and amoxicillin-clavulanic acid (51.5%). Low resistance was seen in the fluoroquinolones, gentamicin and nitrofurantoin. Plasmid curing experiment revealed that most isolates lost their resistance indicating that resistance was borne on plasmids. Plasmid carriage is likely the reason for the high MDR rate of 56.8% observed. **Conclusion:** These findings necessitate the provision of infection control programs which will guide and implement policies.

Key words: Urinary tract infection, biofilm formation/production, *Proteus mirabilis*, plasmid, resistance, multi-drug resistance

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Urinary Tract Infections (UTIs) are one of the most frequent causes of healthcare-associated infection in hospitalized patients worldwide[1]. The UTIs are associated with a significant clinical and economic burden and pose a threat to public health[2]. Indwelling urinary catheters are medical devices originally designed to relieve urinary retention and urinary incontinence in patients for short-term. The long-term use of indwelling catheters is now commonplace in hospital setting and this has resulted in bacterial colonization of urine[3] and increased infection risk. Additionally, catheters can become blocked following the formation of crystalline deposits and encrustations.

Proteus mirabilis, a urease-producing bacterium, often implicated in contamination and colonization, occasionally isolated in severe infections is also one of the most common pathogens involved in complicated urinary tract infections of patients with indwelling urinary catheters[3]. Their role in the production of crystalline deposits and encrustations has been described[4]. Patients on indwelling catheters are predisposed to the development of Catheter-Associated Urinary Tract Infections (CAUTIs) due to the presence of an indwelling catheter device. In most cases, bacteriuria associated with indwelling catheter can turn to a severe infection with manifesting symptoms such as fever, urethritis and cystitis, acute pyelonephritis and bacteremia leading to prolonged morbidity[3].

Proteus mirabilis is a Gram-negative bacterium that is frequently associated with urinary tract infections, especially in patients with indwelling catheters. The ability of the bacterium to cause infection is greatly attributed to its capacity to produce biofilm on both abiotic and biotic surfaces such as indwelling catheters in patients with urinary incontinence. In the process of biofilm formation, several virulent factors are expressed by *P. mirabilis* and these factors comprise adhesion proteins, quorum sensing molecules, lipopolysaccharides, efflux pumps and urease enzymes. Due to its ureolytic biomineralization propensity and the crystalline nature of its biofilms, *P. mirabilis* adheres to catheter surfaces which ultimately leads to the encrustation and blockage the catheter resulting in urine retention and ascending UTIs. In addition, bacteria surrounded in crystalline biofilms become inherently shielded from antimicrobial agents and therefore are generally resistant to conventional antimicrobials as well as being protected from the host immune system[5].

Proteus is a genus in the family of Enterobacteriaceae which consists of rod-shaped Gram-negative bacteria[6]. They are cosmopolitan as they can be found in both soil and aquatic environments as well as in the guts of warm-blooded animals[7]. Biofilms consist of well-organized structures of one or many species of microbial communities in which the cells are permanently fastened to a solid surface and each other. Cells enclosed within the biofilms are surrounded by a self-produced milieu of extracellular polymeric substances that include extracellular polysaccharides, proteins, lipids and DNA[8].

This is particularly more challenging for patients undergoing long-term indwelling urinary catheterization as they usually come down with Catheter-Associated Urinary Tract Infections (CAUTIs). Catheter-associated urinary tract infections are complicated by the crystalline biofilms produced by *P. mirabilis* which over time leads to encrusted and blocked catheters[9]. Affected patients may suffer from urine retention accompanied by painful reflux because of distension of the bladder and pyelonephritis. This condition may in some cases lead to septicemia and endotoxic shock in the affected patients[10].

Most *P. mirabilis* isolates were shown in the past to be susceptible to common antibiotic classes, but in recent times, studies in different countries have indicated that the bacterium is becoming resistant to antibiotics commonly used in the treatment of UTI. Moreso, biofilm formation on catheters, bladder and the urinary tract, could be created by multidrug-resistant organisms. Multidrug resistance (MDR) may be encoded on chromosomes or due to mutations in a resident gene. They may also be located on plasmids, transposons and integrons, leading to the problem of rapid spread which makes managing UTIs more difficult.

There was limited data on the role of *P. mirabilis* in CRUTI in the research location. The current study determined the prevalence of *P. mirabilis* in patients with indwelling catheters, their resistance profile and the location of antibiotic resistant genes.

MATERIALS AND METHODS

Sample and data collection: The study targeted hospitalized patients with an indwelling urinary catheter between the age group of 1 to 60 years. A urine sample was collected by urinary method as per the standard procedure. Positive urine cultures were designated as those with $\geq 10^5$ CFU/mL of a single identified bacterial species. A total of 50 urinary samples were collected from May to August, 2018 from admitted patients on indwelling catheter for a minimum of 3 days in Eku General Hospital, Delta State, Nigeria. Samples were obtained under aseptic condition, properly labelled and transported on ice to the microbiological laboratory for analysis.

Culture and identification: The urine sample were first screened microscopically by Gram's stain before conventional culture techniques were used to isolate the *P. mirabilis* on CLED and MacConkey for 24 hrs at 37°C. The following were used to identify *P. mirabilis*; swarming with characteristic fishy smell, non-lactose fermenting colonies on MacConkey agar, Gram negative pleomorphic bacilli with active motility, positive for phenylalanine deaminase test (PPA), ferments glucose (with acid and gas), sucrose and mannitol but not lactose, reduced nitrate, produced catalase but not oxidase, usually did not utilize citrate, indole negative and a rapid urease producer. Further identification was based on maltose fermentation and the absence of ornithine decarboxylase.

Antibiotic susceptibility testing: Antibiotic susceptibility test was carried out on Mueller-Hinton agar (MHA) by the Kirby Bauer disc diffusion method using the following antibiotic disc; amoxicillin-clavulanate (20/10 µg), ampicillin (10 µg), ceftazidime (30 µg), cefuroxime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ofloxacin (5 µg) and nitrofurantoin (300 µg). The zone size was interpreted after 18-24 hrs of incubation at 37°C following the Clinical and Laboratory Standards Institute (CLSI) Guidelines for 2018 was used to interpret the zones of inhibition[11]. *Escherichia coli* ATCC 25922 was used as control.

Determination of biofilm-forming ability in the isolates: For quantitative detection of biofilm production by the *P. mirabilis* isolates, the method of Harika *et al.*[12] was adopted. Five milliliters of trypticase soy broth was inoculated with the test isolate in a test tube and incubated for 24 hrs at 37°C. To access biofilm production, the broth was decanted and washed with phosphate buffer saline of pH 7.2, air dried and then stained with 0.1% crystal violet for 10 min and finally washed with deionized water. The tube was allowed to dry at room temperature. The experiment was performed in triplicates for all the test isolates with *S. aureus* ATCC 6538P and *E. coli* ATCC 35218 serving as strong and weak biofilm producers, respectively. To score the isolates biofilm production capacity, we considered biofilm as formation of visible layers on walls and the bottom of the tube while non-biofilm producing isolate formed a ringed at the interface of the liquid medium. The levels of biofilm formation was scored as (1) Negative, (2) Weak positive, (3) Moderate positive and (4) Strong positive.

Plasmid curing: Curing of plasmids was performed on isolates by the method of Sijhary *et al.*[13] using a sub-inhibitory concentration of 10% sodium dodecyl sulphate (SDS). Briefly, an overnight broth culture of test isolate was inoculated into 4.5 mL of nutrient broth and incubated. A 0.5 mL SDS (10% concentration) was added and the mixture was incubated at 37°C for 48 hrs. After incubation, 0.5 mL of the broth mixture was inoculated into a freshly prepared 4.5 mL nutrient broth, incubation followed at 37°C for an additional 24 hrs.

Post curing susceptibility test: This was carried on MHA by the Kirby Bauer disc diffusion method as earlier described. The Clinical and Laboratory Standards Institute (CLSI) guidelines for 2018 were followed[11]. The suspension from curing experiment was uniformly spread onto MHA plates. The multi-drug disc containing the same antibiotics as used for antimicrobial susceptibility test was placed using sterile forceps on the agar surface and the plates were incubated at 37°C for 24 hrs.

Ethics approval: The study was approved by the ethics committee of the Faculty of Science, at Delta State University, Abraka. Informed consent was obtained before sample collection.

RESULTS

In the current study, 50 urine samples were collected from patients on indwelling catheters for a minimum of three days and with subjective complaints such as dysuria, urinary frequency and abdominal pain. A total of 37 urine samples were positive for *P. mirabilis*. Of this number, 26 were female while 11 were male. The total aerobic and coliform was determined for microbial population as well as antibiotic susceptibility. Plasmid curing analysis was also carried out on resistant isolates. The mean values of the microbial population as presented in Table 1 range from 3.22×10 to 14.2×10 CFU/mL for total aerobic count and 3.16×10 to 12.74×10 CFU/mL for total coliform count. The prevalence of *P. mirabilis* in relation to age is presented in Table 2. The *P. mirabilis* was most prevalent in the age group 21-35 years with a percentage prevalence of 29.7%. This was followed closely by age group 36-44 (27.0%).

All the isolates were biofilm producers as shown in Table 3. Based on the degree of production, 17 (46%) were moderate producers while 20 (54%) were strong biofilm formers.

The antibiotics-resistant profile before and after plasmid curing was recorded. Resistance profile of the isolates before curing revealed that they were highly resistant to ceftazidime, cefuroxime, ampicillin and amoxicillin clavulanic acid while a low resistance was observed for the fluoroquinolones, gentamicin and nitrofurantoin. However, after curing experiment, most of the *P. mirabilis* isolates lost their resistance indicating resistance being carried on the plasmid as presented in Table 4.

A total of 21 (56.8%) *P. mirabilis* exhibited MDR profile with 14 different resistance patterns. The *P. mirabilis* with MDR to CAZ, CRX, AMP and AUG resistance pattern was most common (Table 5).

Table 1: Total aerobic and coliform count of urine in CFU/mL

		Mean of bacterial counts (log CFU/cm$^5 \times 10^5$)	
Sample in hospital	Source (N = 50) sex/age	TAC (CFU/mL)	TCC (CFU/mL)
1	1-11 M/F	14.20	8.40
2	12-20 M/F	7.30	3.66
3	21-35 M/F	6.30	12.74
4	36-44 M/F	4.92	7.16
5	45-53 M/F	3.22	3.16

TCC: Total coliform count and TAC: Total aerobic count

Table 2: Percentage prevalence of *P. mirabilis* among in-patients in relation to age

Age	Prevalence of isolates per age bracket	Percentage prevalence
1-11	3	8
12-20	7	19
21-35	11	30
36-44	10	27
45-53	6	16
Total	37	100

Table 3: *Proteus mirabilis* strains biofilm forming intensity evaluation using tube-based assay

Degree of biofilm formation	Number of isolates	Biofilm formers (%)
Negative	0	0 (0)
Weak	0	0 (0)
Moderate	17	17 (46)
Strong	20	20 (54)
Total	37	100

Table 4: Antibiotics susceptibility and resistant profile before plasmid curing

Class of antimicrobial agents	Antimicrobial agents	Resistant profile before plasmid curing (N (%))	Resistant profile after plasmid curing (N (%))
Cephalosporin	Ceftazidime (CAZ)	25 (75.8)	3 (17.6)
	Cefuroxime (CXM)	18 (54.5)	2 (11.8)
Penicillin	Ampicillin (AMP)	23 (69.7)	3 (17.6)
Fluoroquinolones	Ofloxacin (OFX)	7 (21.2)	3 (17.6)
	Ciprofloxacin (CPX)	7 (21.2)	3 (17.6)
Aminoglycoside	Gentamicin (GN)	0 (0.0)	1 (5.9)
Nitrofuran	Nitrofurantoin (NIT)	6 (18.2)	4 (23.5)
β-lactam	Amoxicillin clavulanic acid (AMC)	17 (51.5)	3 (17.6)

Table 5: Antimicrobial resistance pattern of the MDR isolates

Number of patterns	Resistance pattern	Number of MDR isolates
1	CAZ, CRX, AMP, CPR, OFL, AUG	3
2	CAZ, CRX, AMP, NIT, CPR, OFL	1
3	CAZ, CRX, AMP, NIT, AUG	1
4	CAZ, CRX, AMP, OFL, AUG	1
5	CAZ, CRX, AMP, NIT, CPR	1
6	CAZ, AMP, OFL, AUG	1
7	CAZ, CRX, NIT, CPR	1
8	CRX, AMP, NIT, AUG	1
9	CAZ, CRX, AMP, AUG	4
10	CAZ, CRX, AMP, CPR	1
11	CAZ, CRX, AMP, OFL	1
12	CAZ, AMP, NIT	1
13	CAZ, AMP, AUG	2
14	CRX, AMP, AUG	2
Total		21

DISCUSSION

The *P. mirabilis* constitutes part of the normal flora of the gastrointestinal tract and thus provides a reservoir for intermittent colonization of the periurethral region. During insertion, urinary catheters are contaminated by the organism and inadvertently transmitted to the urinary bladder[14]. The *P. mirabilis* characterized by its swarming motility is known to be present in many environmental features, such as water, soil and feces-contaminated material[15,16], which may serve as sources of contamination. In this study, a high mean value of microbial population was observed as evidenced in the total aerobic and coliform counts (Table 1). Kishore[17] have reported that the presence of coliform in clinical samples is an indication of high contamination. The *P. mirabilis* is one of the most common causative agents of Urinary Tract Infections (UTIs), particularly in patients with long-term indwelling catheters or under prolonged antibiotic therapy[18]. The medical importance of *P. mirabilis* is not only associated with urinary infection but includes other nosocomial opportunistic infections[19,20]. The prevalence of *Proteus* species as shown in Table 2 reveals that patients between age groups 21-44 were must prone to urinary tract infection by *P. mirabilis*. Moreso, there were more female than male on catheter in this study; 26 versus 11 (Table 3). This result agreed with the findings of Jamil *et al.*[1], who reported that UTIs were more common in women of ages 20 to 50 years. Other studies in Nigeria, also reported a higher isolation of *P. mirabilis* in urine[21,22].

The ability to form unusual biofilms which are crystalline in nature is a characteristic of *P. mirabilis*. This plays a major role in the encrustation and obstruction of indwelling catheters thereby causing complications in CAUTIs. Urease and capsule polysaccharides are the two virulence factors known to play a central role in the formation of *P. mirabilis* crystalline biofilms[23]. In this study, all the isolates were biofilm producers with 46% moderate producers while 54% were strong biofilm formers. This finding was very much similar to those of Maione *et al.*[24] and Fusco *et al.*[25], who reported above 60% biofilm producing capacity among *P. mirabilis* isolated from UTIs and CAUTIs in Italy and clinical isolates, respectively. The capacity for biofilms production is significant in the pathogenesis of microbial infections as it plays a crucial role in pathogens' ability to resist antimicrobial agents resulting in persistent causes of chronic infections. Since biofilm helps in bacterial adaptation to adverse environmental factors, it therefore plays significant implications for public health. Bacteria embedded within the biofilm are generally more resistant to antimicrobial agents than the planktonic counterparts thus hindering therapies with antimicrobial agents. This inherent ability of uropathogens to form biofilm plays a critical role in their being able to persist in the urinary tract and associated catheters, this encrustation helps them to evade the immune system, engenders recurrent infections as well and increases the emergence of multidrug resistance in them. Fusco *et al.*[25] have highlighted the role of biofilm formation in UTIs.

Evidently, *P. mirabilis* can lead to a high rate of infections in populations[26,27], necessitating the need to better understand the resistance and MDR profile in other to control infections. Due to the inherent ability of *P. mirabilis* to form biofilm, the infections they cause are generally more severe and more difficult to treat than those of *E. coli* as the biofilm in them aids in antimicrobial resistance of the organism. The level of antibiotic resistance in any region depends on the stewardship of their usage in the area[28-32]. The antimicrobial resistant pattern revealed that *P. mirabilis* was most resistant to the beta lactams, especially ceftazidime. Algammal *et al.*[33] also observed a high level of ceftazidime resistance in *P. mirabilis*. Low antibiotic permeability occasioned by mutations in porin and protein of the outer membrane might have contributed to the resistance of cephalosporin in the *P. mirabilis* isolates[34]. This may also be because cephalosporins are widely prescribed in the treatment of urinary infections[35]. Various studies have provided evidence showing that the inappropriate and unregulated use of antibiotics as well as inadequate infection control practices are responsible for the emergence and reemergence of resistant bacteria[30,36,37]. Low resistance was observed in the fluoroquinolones and nitrofurantoin in this study. However, some studies have observed that nitrofurantoin is a treatment option for the treatment of uncomplicated lower urinary infections[38,39].

Plasmids are extrachromosomal genetic elements that codes for among many other things resistance to antimicrobial agents as bacteria habourning a resistance plasmid often overcome the selective pressure occasioned by increased use and indiscriminate abuse of antimicrobial agents[40]. Antimicrobial resistances mediated by plasmid encoded genes have been reported extensively across the globe[40-42]. The antibiotic susceptibility of isolates after plasmid curing revealed that most isolates lost their resistance indicating that resistance was borne on plasmids (Table 4), which explains the high rate of MDR observed in this study. Of the 37 *P. mirabilis* isolated 21 (56.8%) exhibited MDR profile with 14 different resistant patterns (Table 5). Consequently, empiric therapy is likely to fail.

Lakhundi and Zhang[43] and Mirzaei *et al.*[44] have revealed a high prevalence of MDR *P. mirabilis*. The MDR in bacteria is generally due to multiple acquired plasmids, transposons and or intergrons bearing genetic determinants for different mechanisms of resistance. However, *P. mirabilis* carries a myriad of virulent factors including antibiotic-resistance genes with the potential to transfer antibiotic resistance to other strains due to harbouring a functional and integrative element called ICEPmu1[45]. This facilitates the spread and persistence of the MDR *P. mirabilis* among various bacterial strains. The implication of this is that under prevalent antimicrobial selective pressure in the hospital environment, advantageous mutation and the opportunity to acquire additional resistance genes will abound.

The limitation of this study was the lack of a modern technique, due to limited financial sources.

CONCLUSION

The study has shown that the use of an indwelling catheter is associated with urinary tract infection and MDR. Resistance was harboured more on plasmids facilitating spread of resistance. There is a need to put in place infection country programs in Nigeria as it is lacking. Such programs will implement and monitor policies and practices to minimize infections associated with the use of these devices. This will also go a long way in reducing bacteremia and MDR.

SIGNIFICANCE STATEMENT

In this study, we assess the prevalence of *P. mirabilis*, its biofilm forming capability, associated antimicrobial resistance of the isolates and the level of multidrug resistance among them. We report that all the isolates of *P. mirabilis* were biofilm formers and that the levels of antimicrobial resistance and multidrug resistance among them are relatively high. Therefore, an effective preventive control program is required to reduce the spread of the pathogen as well as stymie the spread of antibiotic resistance.

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