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Brief communication (Original)

Multidrug resistance by biofilm-forming clinical strains of *Proteus mirabilis*

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Background: Biofilm formation on indwelling devices is one of the most important mechanisms playing a role in device-related urinary tract infections caused by *Proteus mirabilis*. Increasing antibiotic resistance of microorganisms has raised questions concerning the relationship between biofilm formation and drug resistance.
Objective: To determine clinical prevalence, antibiotic resistance of, and biofilm formation by *P. mirabilis* strains.
Methods: We studied the susceptibilities of various *P. mirabilis* strains isolated from different clinical materials by a disc-diffusion method. Biofilm formation was determined by a crystal violet binding assay.
Results: Two (13%) of 15 *P. mirabilis* strains were found to be strong biofilm formers (SBF). Both SBF *P. mirabilis* strains were isolated from urine samples from children less than 15 years old in a pediatric emergency unit. Cefixime, cefazolin, ceftriaxone, amikacin, and piperacillin/tazobactam were the most effective antibiotics against 15 *P. mirabilis* strains (100%), whereas SBF *P. mirabilis* strains were multidrug resistant or (resistant to 5 different antimicrobial classes). Both of the SBF *P. mirabilis* strains, but neither of the weak biofilm forming *P. mirabilis* strains were resistant to ampicillin and ceftazidime among β-lactam antibiotics, or tobramycin and gentamicin among aminoglycoside antibiotics used in the present study.
Conclusions: Children comprise the only patients infected with SBF *P. mirabilis* strains and both SBF P. mirabilis strains displayed high antimicrobial resistance in our setting.

Keywords: Biofilm formation, device-related infections, multidrug resistance, *Proteus mirabilis*, urinary tract infection

Proteus mirabilis, a highly motile microorganism, is a member of the Enterobacteriaceae family and is responsible for many clinical infections including those of the urinary tract, abdominal cavity, blood stream, and indwelling devices including vascular access ports, scleral buckles, ureteral stents, urethral catheters, and tracheoesophageal fistulas [1-4]. Among these clinical infections, *P. mirabilis* strains are more commonly associated with urinary tract infections; particularly in patients undergoing urinary catheterization and having urolithiasis [1]. One of the most important mechanisms playing a role in indwelling device-related urinary tract infections, is known as biofilm formation [3]. Once *P. mirabilis* adheres to living tissue or nonliving surfaces, it may form a slimy layer known as a biofilm. The biofilm protects these microorganisms from the host defense system and from antibiotics; often leading to repeated infection by *P. mirabilis* [1]. Moreover, *P. mirabilis* generates ammonia and elevates the pH of the urine to >7.2, promoting precipitation and aggregation of struvite or apatite crystals [5, 6]. These crystals are deposited directly onto the catheter surface or into microbial biofilms; leading to blockage and encrustation of catheters, retention of urine in the bladder and development of bacteriuria [2, 6]. Previous reports showed a correlation between biofilm production and multiple drug resistance in clinical isolates [7-8]. Increased antibiotic resistance is thought to be related to biofilm formation. This increased resistance is related to gene transfer within biofilms [9]. The aim of this study was to determine the clinical prevalence of *P. mirabilis* strains in our setting, their antibiotic resistance, and tendency to form biofilms.

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Materials and methods

Bacterial strains

Fifteen strains of *P. mirabilis* strains were isolated anonymously from urine, feces, and abscesses from various adult and pediatric services at a hospital in

Ankara. Isolated *P. mirabilis* strains were inoculated into brain–heart infusion broth media, which includes 10% glycerol, and stored at –20°C until further analysis. Isolates were identified by standard phenotypical methods [10], and identification was further confirmed by using a Vitek-32 system (BioM□rieux, Marcy-l'□toile, France).

Antibacterial susceptibility testing

The susceptibility of the isolated *P. mirabilis* strains to amoxicillin/clavulanic acid (AMC) (20/10 µg), ciprofloxacin (CIP) (5 µg), gentamicin (CN) (10 µg), trimethoprim/sulfamethoxazole (STX) (1.25 µg), nitrofurantoin (F) (300 µg), clindamycin (CC) (2 µg), amikacin (AK) (300 µg), ampicillin (AM) (10 µg), imipenem (IPM) (10 µg), piperacillin/tazobactam (TZP) (100/10 µg), cefazolin (CAZ) (30 µg), cefixime (CFM) (5 µg), ceftazidime (CZ) (30 µg), ceftriaxone (CRO) (10 µg) was assessed by disc-diffusion methods according to National Committee for Clinical Laboratory Standards (NCCLS). The strains were classified as resistant (R), intermediate (I), or sensitive (S), according to the zone table published by the Clinical and Laboratory Standards Institute (940 West Valley Road, Suite 1400, Wayne, PA, USA).

Biofilm formation

The crystal violet binding assay described by O'Toole was used with some modifications to determine biofilm formation by *P. mirabilis* strains [11]. Briefly, bacterial cells were inoculated into brain–heart infusion broth medium and subsequently incubated at 37°C overnight. The overnight culture was diluted 1:100 and the wells of a polystyrene plate were filled with diluted inoculum. Then, the plates were incubated for 48 h at 37°C. Following this, the wells were washed with distilled water, dried, and then stained with 1% crystal violet for 45 min at room temperature. Finally, after washing the wells again and waiting for them to dry, bound crystal violet in each well was solubilized by ethanol–acetic acid (90:10) solution and the crystal violet in solution from each well was determined using a spectrophotometer at 540 nm. The experiments were performed in triplicate. Strains having an optical density (OD) ≥0.1, were identified as biofilm producers and classified into 3 categories as follows:

$0.1 \leq OD < 0.4$ Weak Biofilm Former (WBF)
$0.4 \leq OD < 0.8$ Intermediate Biofilm Former (IBF)
$OD \geq 0.8$ Strong Biofilm Former (SBF)

Results

P. mirabilis strains were obtained from 3 different clinical materials including; urine, feces and abscess, with urine the most common source of *P. mirabilis* (11/15 or 73%) **(Figure 1)**.

The pediatric emergency unit and general pediatrics departments had the highest prevalence (8/15) of *P. mirabilis* strains **(Figure 2)**.

Additionally, the age interval at which *P. mirabilis* infections occurred most frequently, was observed as ≤15 years (9/15 or 60%) **(Figure 3)**.

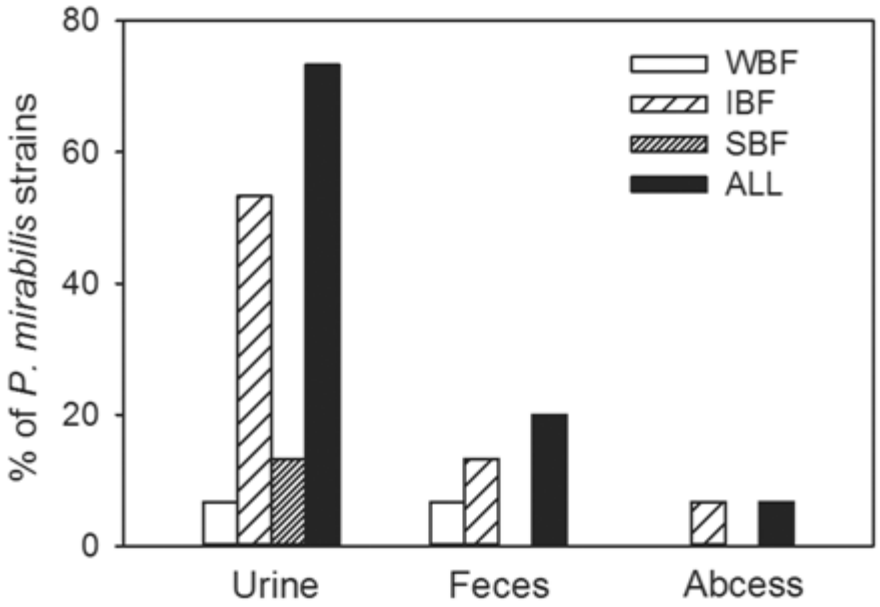

Figure 1. Percentage of weak biofilm forming (WBF, 2 total), intermediate biofilm forming (IBF, 11 total), strong biofilm forming (SBF, 2 total) and all *P. mirabilis* strains (ALL) in various clinical materials.

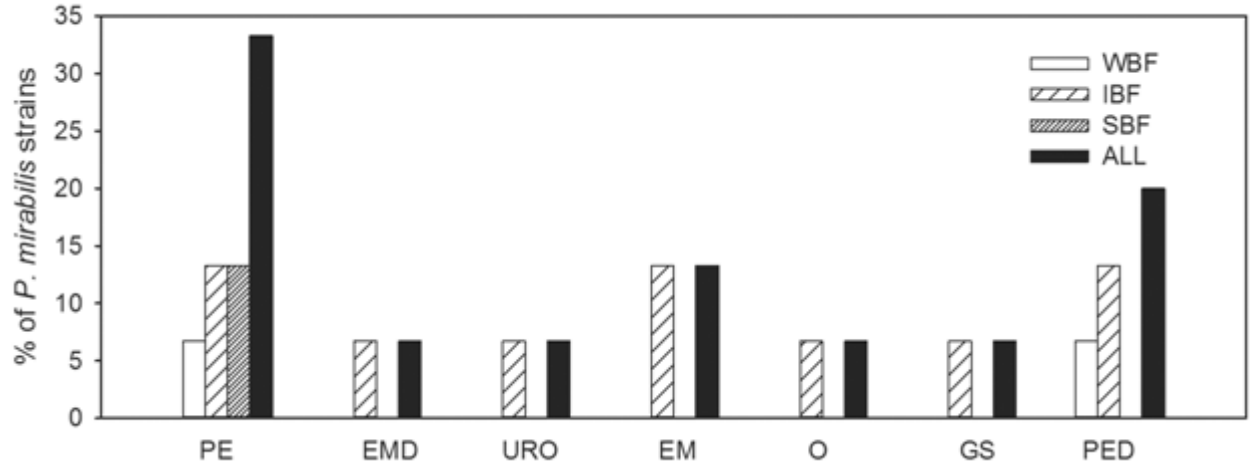

Figure 2. Percentage of weak biofilm forming (WBF), intermediate biofilm forming (IBF), strong biofilm forming (SBF), and all *P. mirabilis* strains in different service units.
(PE = pediatric emergency (5 total), EMD = endocrine and metabolic diseases (1 total), URO = urology (2 total), EM = emergency medicine (2 total), O = otorhinolaryngology (1 total), GS = general surgery (1 total), PED = pediatrics (3 total)

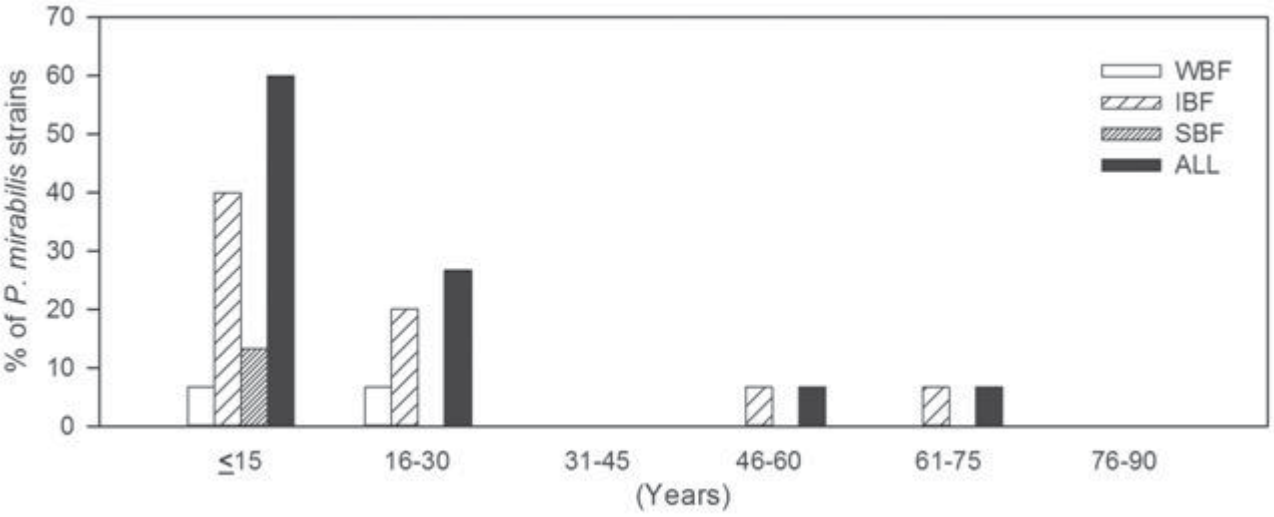

Figure 3. Percentage of weak biofilm forming (WBF), intermediate biofilm forming (IBF), strong biofilm forming (SBF) and all *P. mirabilis* strains (ALL) in individuals of various ages. (16–30 y, 4 strains total; 46–60 y and 61–75 y, 1 strain each)

After application of the crystal violet binding assay for biofilm screening, *P. mirabilis* strains were classified into 3 categories according to their biofilm formation. When the occurrences of strong, intermediate, and weak biofilm forming *P. mirabilis* isolates in different clinical parameters were examined, intermediate biofilm forming *P. mirabilis* strains were isolated from all clinical materials and service units. However, strong biofilm forming strains were only isolated from samples of urine **(Figure 1)** from the pediatric emergency department being from patients aged <15 years **(Figures 2 and 3)**.

Antibiotic sensitivity testing of *P. mirabilis* strains against 15 different antibiotics revealed that all 15 strains tested were sensitive to third generation cephalosporins (cefixime, cefazolin, and ceftriaxone) **(Figure 4)**.

However, all 15 strains were resistant to clindamycin and 14 (93%) of all 15 *P. mirabilis* strains were resistant to nitrofurantoin. Moreover, both of the SBF *P. mirabilis* strains, but neither of the WBF *P. mirabilis* strains, were resistant to ampicillin and ceftazidime among β-lactam antibiotics, and tobramycin and gentamicin among aminoglycoside

antibiotics. SBF *P. mirabilis* strains were the only strains showing resistance to ciprofloxacin **(Figure 4)**.

We found that most *P. mirabilis* strains, both of the SBF *P. mirabilis* strains, most of the 11 IBF strains, and 1 of the 2 WBF *P. mirabilis* strains were resistant to 3 or more classes of antibiotics and defined as multidrug resistant (MDR) (**Table 1**). Additionally, both SBF *P. mirabilis* strains were resistant to 5 different classes of antibiotics. Furthermore, both SBF *P. mirabilis* strains showed resistance to at least 1 antibiotic tested from each of the lincosamide, furan, aminoglycoside, and β-lactam classes **(Figure 4)**.

Discussion

The characteristics of *P. mirabilis* strains pose a common problem in the management of infections. By means of providing slow and protected bacterial growth, poor penetration of antimicrobials, interbacterial transfer of resistance genes via plasmids and conversion, biofilms are regarded as responsible for most recurrent and persistent nosocomial infections [12, 13]. Formation of biofilms on urethral catheters, continuous ambulatory peritoneal and intravenous hemodialysis tubing, influences the incidence and outcomes of infections associated with urinary tract manipulation [12]. Moreover, complicating renal calculi are another common source of biofilm formation and chronic infections [12].

Urine was the most common clinical material from which *P. mirabilis* were isolated **(Figure 1)** [13, 14]. Blood stream infections caused by *P. mirabilis* can cause mortality [4, 15]. We found a high proportion of *P. mirabilis* strains in patients aged 15 years or younger in pediatric emergency and general pediatrics units **(Figures 2 and 3)**. This is consistent with the suggestion that infants and young children may not yet have a fully developed defense system [16], and/or that the swarming ability of *P. mirabilis* strains may cause migration, aided by children's behavior, and fecal contaminations of the urinary tract.

When biofilm formation of *P. mirabilis* was examined; 2 strains were found as SBF and both of these strains were isolated from urine samples of patients aged <15 years showing that biofilm formation-related urinary tract infections in children is a significant hazard to their health. That biofilms are formed readily on the surface of indwelling catheters

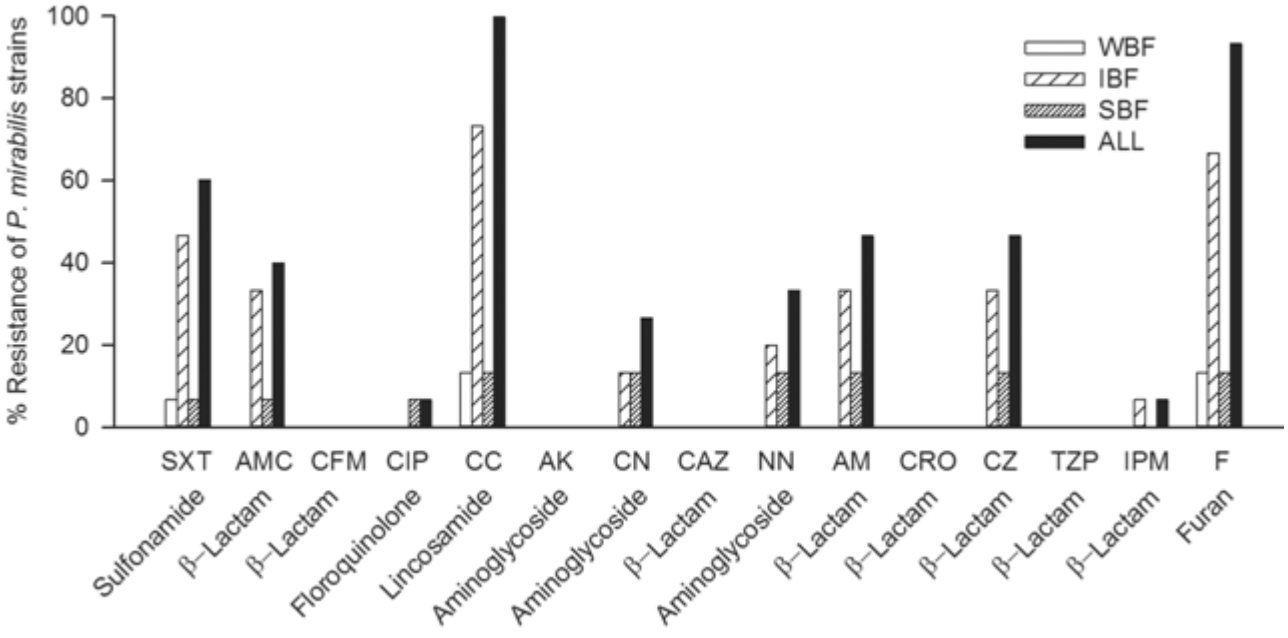

Figure 4. Percentage of weak biofilm forming (WBF), intermediate biofilm forming (IBF), strong biofilm forming (SBF) and all *P. mirabilis* strains (ALL) displaying resistance to 15 different antibiotics in various classes of antibiotics (SXT = trimethoprim/sulfamethoxazole, AMC = amoxicillin/clavulanic acid, CFM = cefixime, CIP = ciprofloxacin, CC = clindamycin, AK = amikacin, CN = gentamicin, CAZ = cefazolin, NN = tobramycin, AM = ampicillin, CRO = ceftriaxone, CZ = ceftazidime, TZP = piperacillin/tazobactam, IPM = imipenem, F = nitrofurantoin)

and on kidney stones is a major cause of persistent urinary tract infections [12, 17, 18]. The incidence of kidney stones in children shows an increasing trend in reports from the USA, Turkey, and Thailand. This increased incidence of kidney stones is thought to be related with the increased frequency of isolation of SBF *P. mirabilis* strains from children [19-21].

When antimicrobial resistance of *P. mirabilis* strains is examined; clindamycin, a member of the lincosamides, was the only antibiotic to which all *P. mirabilis* strains showed resistance **(Figure 4)**. *P. mirabilis* strains are usually naturally resistant to the lincosamide class of antibiotics [20]. Additionally, the majority of *P. mirabilis* strains were found to be resistant to nitrofurantoin **(Figure 4)** [22, 23]. Apart from these, all third generation cephalosporins (cefazolin, cefixime, and ceftriaxone), which are members of the β-lactam group, and antibiotics such as piperacillin/tazobactam, and amikacin were seen as the most efficient antibiotics against *P. mirabilis* strains **(Figure 4)**. However; increasing resistance toward broad-spectrum cephalosporins and fluoroquinolones in clinical isolates of *P. mirabilis* is reported [13, 22]. Among fluoroquinolones; ciprofloxacin was the only antibiotic used in this study and interestingly, all ciprofloxacin resistant *P. mirabilis* strains were observed as SBF **(Figure 4)**. A new plasmid-mediated quinolone resistance gene *qnrC* has a role in ciprofloxacin resistance of *P. mirabilis* [24]. The reason for increased ciprofloxacin resistance in SBF strains may well be related to interbacterial transfer of resistance genes via plasmids within biofilms [9, 18, 25]. Our study supports this concept because both of our SBF *P. mirabilis* strains were found to be MDR **(Table 1)**.

Additionally, all SBF *P. mirabilis* strains were resistant to tobramycin and gentamicin among aminoglycosides, and ampicillin and ceftazidime among β-lactams, whereas neither of the WBF *P. mirabilis* strains were resistant to any antibiotics belong to the aminoglycoside (tobramycin, gentamicin, and amikacin) or β-lactam (ampicillin, ceftriaxone, ceftazidime, piperacillin/tazobactam, imipenem, amoxicillin/clavulanic acid, cefixime, cefazolin) antibiotic classes **(Figure 4)**.

Acquisition of plasmid mediated AmpC β-lactamases is an important reason for the increase in extended-spectrum β-lactamase (ESβL) producing *P. mirabilis* strains in various geographical locations [26]. Interestingly, ESβL-positive *P. mirabilis* strains have been reported as coresistant to aminoglycoside and fluoroquinolone classes of antibiotics [3]. In parallel with this, ampicillin and ceftazidime (β-lactam group antibiotics) resistant SBF *P. mirabilis* strains were found as coresistant to tobramycin and gentamicin among the aminoglycoside class of antibiotics, and an ampicillin and ceftazidime resistant SBF *P. mirabilis* strain was found as coresistant to ciprofloxacin among the fluoroquinolone class of antibiotics. Considering these findings, we believe that the β-lactam resistance of SBF *P. mirabilis* strains to ampicillin and ceftazidime may result from the presence of plasmid mediated AmpC β-lactamase.

Conclusion

The present study shows that children comprise the only group of patients infected with MDR and SBF *P. mirabilis* strains. *P. mirabilis* strains appear to emerge as important pathogens in the urinary tract infections of children, and this may be related to their SBF ability, which may contribute to their MDR. Moreover, finding a relationship between biofilm forming *P. mirabilis* strains and MDR, suggests that gene transfer mechanisms within the biofilm environments are likely.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Table 1. Percentage of *P. mirabilis* strains displaying resistance to 5 different classes of antibiotics and percentage of multidrug resistant *P. mirabilis* strains in each biofilm group.

	WBF *P. mirabilis* strains (%)	**IBF *P. mirabilis* strains (%)**	**SBF *P. mirabilis* strains (%)**
Multidrug resistance	1/2 (50)	7/11 (63)	2/2 (100)
Resistance to 5 different classes of antibiotics	0	2/11 (18)	2/2 (100)

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