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Antibiotic Resistance Pattern among Biofilm Producing and Non Producing *Proteus* Strains Isolated from Hospitalized Patients; Matter of Hospital Hygiene and Antimicrobial Stewardship

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Abstract: A retrospective study on antimicrobial susceptibility and biofilm production were carried out for eighty eight strains of *Proteus* strains isolated from UTI and other hospital samples during April 2011-April 2012. The antibiotic susceptibility was carried out by Kirby-Bauer disk diffusion and MIC by E-test. Biofilm production was measured by microtiter method and confirmed by Scanning electron microscopy. Plasmids from biofilm producing isolates were detected by alkaline lysis technique. From 88 patients infected by proteus species, 58% were female and 42% were mail. The most frequent age range was 20-29 (77.39%) and the least were 60-69 years old (3.4%) ($p = 0.05$). Eighty one isolates were identified as *P. mirabilis* while, 7 identified as *P. vulgaris*. 67.04% [n = 59] of the isolates showed MIC range ($16\text{-}32\pm0.05$ μg mL^{-1}) to ceftriaxone, 46.59% [n = 41] exhibited least MIC range to chloramphenicol ($8\text{-}64\pm0.08$ μg mL^{-1}). 31% [n = 28] of the isolates also exhibited MIC range 1-4 μg mL^{-1} to ciprofloxacin. 17% [n = 15] of the isolates exhibited strong biofilm while, 6% [n = 6] did not show any biofilm ($p\leq0.05$). Plasmid isolation from biofilm producing isolates revealed that stains number 19, 24 and 87' that produced strong biofilm carried similar high M. Wt. plasmid. From above results it can be concluded that the majority of Proteus isolated from UTI patients were belong to *P. mirabilis*. Ciprofloxacin was the most effective antibiotic for treatment of the infected patients. Limited number of the isolates could produce strong biofilm that were bearing plasmids. Majority of the biofilm producing isolates were also resistance at least to 4 antibiotics routinely prescribed in our hospital.

Key words: *Proteus*, antibiotic resistance, MIC, biofilm, plasmid

INTRODUCTION

Gram negative bacteria especially *E. coli*, *Klebsiella*, *Serratia* and *Proteus* are important source of hospital associated infection in many parts of the worlds. The important features of these bacteria are resistance to different antibiotics which their genes usually carried on large molecular weight plasmids (Lied, 2011; Khan and Musharraf, 2004). Plasmids not only play important role in antibiotic resistance phenomenon but also they probably can mediate the biofilm formation using component of signal transduction system (Hola *et al*., 2012; Tenke *et al*., 2006; Ghigo, 2001). Production of biofilm enables bacterium to attach to hospital devise like catheter and withstand the antimicrobial activity of various antibiotics (Saint and Chenoweth, 2003; Sharp *et al*., 1974). *Proteus mirabilis* is main species that can cause wide range of infections including urinary tract, blood and burn (Mozafari Nia *et al*., 2011). This organism was often associated with Urinary Tract Infections (UTI) in patients carried mechanical devices such as catheter (Jacobsen and Shirtliff, 2011; Stickler, 2008). Urinary tract infections are very painful and can become lethal if the infection spreads to other organ in the body (Warren *et al*., 1987). Antibiotic resistant phenotypes of *P. mirabilis* were reported by various authors (Coker *et al*., 2000). In one study, Sabbuba *et al*. (2002) examined the ability of organisms to stick to hospital devices. It was found that, the swarmer cells of Proteus mirabilis migrated over all four types of catheter such as hydrogel-coated latex, hydrogel/silver-coated latex, silicone-coated latex and silicone.

Ability of *P. mirabilis* to attach in the hospital devices is mainly due to biofilm production in this organism. It is reported that biofilms developed by *P. mirabilis* is increasing source of catheter associated UTI infection in the hospitals (Stickler *et al*., 1993). Majority of patients also exhibited ascending urinary tract infection and pyelonephritis (Hochreiter *et al*., 2003; Stickler and Morgan, 2006).

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It was known that treatment of catheter associated UTI was very difficult and recurrent infection may occur in 62% of the patients (Absalon *et al*., 2012).

P. mirabilis not only can produce infection in urinary tract but also it can cause super infection in severely burn patients. Biofilm formation and antibiotic resistance play important role in this phenomenon too (McManus *et al*., 1982). Khan and Musharraf (2004) studied drug resistance pattern and plasmid content of hospital isolates of *P. mirabilis*. It was found that that this infection is more common in young pregnant women. Catheter associated biofilm formation by *P. mirabilis* found to be in both motile wild type and non motile mutant have same ability to stick on the surface of catheter (Jones *et al*., 2007).

Multiple antibiotic resistant *P. mirabilis* has created serious concern in treatment of catheter associated UTI infections. The colonization of catheters by biofilm producing strains of *P. mirabilis* and easy excess to urinary tract caused treatment failure due to antibiotic resistance biofilm producing strains.

The aim of this investigations were; to isolate *Proteus* species from hospitalized patients, to study biofilm production and antibiotic sensitivity of the isolates, finally to detect plasmid from those isolates that show strong biofilm.

MATERIALS AND METHODS

Sampling and bacterial identification: Eighty eight strains of proteus strains were collected from urinary tract and other sites patients hospitalized in different hospitals in Kerman, Iran during April-2011 to April-2012. The sample size was selected according to published paper (Mozafari Nia *et al*., 2011). The sample from urinary tract catheter was obtained by scraping of the biofilm with the help of sterile blade and inoculated in to 2 mL sterile Stuart Transport (ST) medium while, the urine samples were obtained from mid portion of urine and centrifuged. 0.1 mL of the lower part was then inoculated into sterile ST medium. Both the samples were transferred to the department of microbiology for further analysis within 1 to 2 h of sampling. In case of burn and pulmonary infections, the samples were collected with the help of sterile swabs and inoculated into 1 mL ST medium as previously suggested. A loopful of the bacterial culture was suitably diluted (10^{-2}) with sterile 0.1 N normal saline and streaked onto MacConkey and sheep blood agar medium (Merck, Germany), the plates incubated for 24 h at 37°C. Bacterial identification were performed by routine microbiological tests such as gram reaction, motility, ability to ferment lactose, H_2S, Urease production, Phenyl Alanine Deaminase (PAD), Lysine Decarboxylase (LDC), MR, VP and Indole tests. The identified isolates were mixed with 40% glycerol in True North^{TM} Cryogenic Vials (TNC) containing 1mL sterile Trypticase Soy Broth (TSB) and preserved at -70°C for further investigation.

Antibiotic sensitivity tests: Preliminarily antibiotic sensitivity of the above isolates was determined by Kirby-Baur disk diffusion break point method using following antibiotic disks; Ciprofloxacin (Cf) [5 μg/disk], Amikacin (AK) [30 μg/disk], Kanamycin (K) [30 μg/disk], Gentamicin (Gm) [10 μg/disk], Cefotaxime (CE) [30 μg/disk], Ceftrixone (CI) [30 μg/disk], Cefazoline (CZ) [30 μg/disk] and Chloramphenicol (C) [30 μg/disk]. All antibiotic disks were purchased from Padtan-Teb (Tehran, Iran). 0.1 mL of each proteus isolate at 1×10^{8} CFU mL^{-1} were inoculated into Muller-Hinton agar (MHA) [Hi-media, India] and spread throughout medium with the help of a sterile swab. The antibiotic disks (five disks in each plate) were then kept on the surface of each plate and incubated at 37°C for 24 h. The zone of inhibition surrounding each disk was measured and labeled as resistance, intermediate, sensitive according to CLS procedure (CLSI, 2009). Minimum Inhibitory Concentration (MIC) of above antibiotics against the isolates was carried out by E-test. Inoculated plates were allowed to dry before E-test strips were applied to the medium. E-test inoculum preparation and plating, strip application and subsequent MIC determinations were carried out in accordance with the manufacturer's instructions and CLSI guidelines (CLSI, 2009). *P. mirabilis* ATCC 29906 was included as a control strain for susceptibility testing.

Biofilm production by microtiter plate method: The biofilm production of the above Proteus isolates was determined by microtiter method as described previously (Stepanovic *et al*., 2007). Briefly, one loopful of colony from proteus isolates was inoculated into a 2 mL sterile TSB medium containing 1% v/v glucose to optimize biofilm production. Optical Density (OD) was adjusted to 650 nm (10^{6} CFU mL^{-1}) and with final dilution (1:40). One hundred micro liter of the each bacterial growth was added to two parallel Elisa-Reader wells (TeCan-Austria). Similarly, 100 μL of the medium was added to the two well without any bacterium (negative control). The microtiter plate was kept under static condition. After 24 h at 37°C, no adherent cell suspensions were aseptically aspirated and replaced with 10 µL 0.1 N sterile phosphate buffer (pH-7.5). One hundred and fifty micro liter concentrated methanol was then added to each well and kept at room temperature (24°C) for 10 min in order to fix the biofilm. The methanol was slowly removed and replaced with 200 µL of 1% crystal violet dye. The plates containing biofilm matrix then washed slowly with tape water and kept at room temperature till dried. To this preparation, 160 µL glacial acetic acid (33% v/v) was added and the optical density of each well was measured at 570 nm. Duplicate set was run at a time.

Biofilm detection by scanning electron microscope (SEM): SEM analysis was done according the method described previously (Pour *et al*., 2011). Briefly, One loopful growth of the biofilm producing isolates from TSB medium on Elisa-Reader wells was transferred aseptically into sterile petriplate containing 10 mL glutraldehyde (10% v/v) in double distilled water at different time intervals (8, 16 and 24 h) and kept at 4°C overnight. The samples were mounted on the standard specimen stubs and then placed on the microscopic grids. The grids were coated with thin layer of gold. Samples were observed with magnification of EHT 10.000 (11 WD) using Scanning Electron Microscope (Philips-Holland). The experiment was repeated twice to check the genuinety of the biofilm formation. The micrograph of the each sample at different time interval was recorded by a camera attached to the high resolution recording unit. A negative control consist of an isolate with no biofilm was taken along the tests experiment.

Plasmid isolation from biofilm producing isolates: Plasmids from biofilm producing *Proteus* strains were isolated by Birnboim and Doly alkaline lysis technique (Birnboim and Doly, 1979) and observed on 0.7% agarose gel. Electrophoresis was conducted for 4 h at 60 volt (35mA) using 500 mL Tris-Borate-EDTA (TBE) buffer (pH-8.3) and plasmid bands were photographed by a camera attached UV gel documentation system (UV Tech-Cambridge) after stained with 0.5 µg mL^{-1} ethidium bromide.

Statistical analysis: The difference in susceptibility patterns was analyzed by the Chi-square or two-tailed Fisher exact test. The significance of the biofilm form by eighty eight isolates were analysed by using a one-way analysis of variance (ANOVA). A $p<0.05$ was considered as statistically significant.

RESULTS

Bacterial distribution: Retrospective distribution of *Proteus* strains among eighty eight hospitalized patients infected by this bacterium according to age during showed that. Overall, 37 (42%) were male and 51 (58%) were female (Table 1). The most frequently infected patients by proteus species were in the range of 20-29±0.08 and least people infected were in the range of 60-69±0.02 years old. 92.2% of the isolates were collected from urinary tract and remaining 5.6% isolated from burn patients and 2.2% from the other body sites (p = 0.05). In this study only seven isolates were belong to *P. vulgaris* and remaining were all belong to *P. mirabilis*. The colonies on MacConkey agar were circular, smooth, convex, translucent, mucoid, nonpigmented and the lactose utilization test for all isolates was negative. They were gram-negative short rod, highly motile and exhibited swarming on both MacConkey and bloodagar medium.

Antibiotic sensitivity: The results of the antibiotic susceptibility of the above isolates are shown in Table 2 and 3. The results revealed that, 67% (n = 59) of the isolates were highly resistant to ceftriaxone with MIC

Table 1: Distribution of *Proteus* strains isolated from patients in Kerman hospitals according to ages

Age (years old)	Number	Infected (%)
9-1	10	11.36
19-10	15	17.04
29-20	35	39.77
39-30	12	13.36
49-40	6	6.18
59-50	7	7.59
69-60	3	3.40
Total patients	88	100.00
Mean±SD = 39±0.07		

Table 2: Antibiotic susceptibility of eighty eight *Proteus* strains isolated from patients to eight antibiotics routinely used in the hospital for treatment of UTI patients

Antibiotic	Sensitive		Intermediate		Resistant	
Disk	No.	%	No.	%	No.	%
C	47	53.40	21	23.36	20	22.72
K	39	44.31	23	26.13	26	29.54
Gm	35	39.77	16	18.18	37	40.04
Cf	41	46.59	19	21.95	28	31.81
CE	41	46.59	16	18.18	31	35.22
CI	29	32.95	19	21.95	40	45.45
CZ	30	34.09	11	12.50	47	53.40
AK	36	40.90	11	12.50	41	46.59

Muller-Hinton agar was used for susceptibility testing, Inoculum diluted to obtain 1×10^{8} CFU mL^{-1}, Ciprofloxacin (Cf) [5 µg/disk], Amikacin (AK) [30 µg/disk], Kanamycin (K) [30 µg/disk], Gentamicin (Gm) [10 µg/disk], Cefotaxime (CE) [30 µg/disk], Ceftrixone (CI) [30 µg/disk], Cefazoline (CZ) [30 µg/disk] and Chloramphenicol (C) [30 µg/disk] range 16-32 μg mL^{-1} while majority of the isolates were sensitive or intermediate to ciprofloxacin with MIC range 1-4 μg mL^{-1}. 29.54% of the isolate were resistant to kanamycin while, 46.59% were resistant to amikacin with MIC range 4-64 μg mL^{-1} (Table 3). The isolates also exhibited high degree of resistance to cefotaxime with MIC range 2-64 μg mL^{-1}. The majority of *P. mirabilis* isolates tolerated concentrations exceeding 64 μg mL^{-1} of antibiotics from third generation of cephalosporins while, MIC to gentamycin did not exceed 16 μg mL^{-1} as shown in Table 3.

Biofilm production: Biofilm quantification of proteus isolates revealed that 6.8% (n = 6) of the isolates did not show any biofilm while, 36% (n = 32) exhibited weak, 39.7% (n = 35) showed intermediate biofilm and 17.4% (n = 15) demonstrated strong biofilm activity. Quantification of biofilm production among proteus strains are shown in Fig. 1. The results were further confirmed by SEM technique as shown in Fig. 2a-c. The

Table 3: Minimum inhibitory concentration (MIC) of o eight antibiotics against eighty eight strains of *Proteus* that isolated in this study

	MIC (μg mL^{-1})		
Antibiotic	MIC range	MIC_{50}	MIC_{90}
C	8-64	16.0	64
CF	1-4	0.5	4
AK	4-64	16.0	64
Gm	4-16	4.0	16
K	8-32	8.0	32
Ce	2-64	8.0	64
CI	16-32	16.0	32
CZ	4-32	8.0	32

MIC was measured by E-test, The inoculum concentration was adjusted to 1×108 CFU mL^{-1}, The difference in susceptibility patterns (MIC_{50} and MIC_{90} was analyzed by the Chi-square and two-tailed Fisher exact test

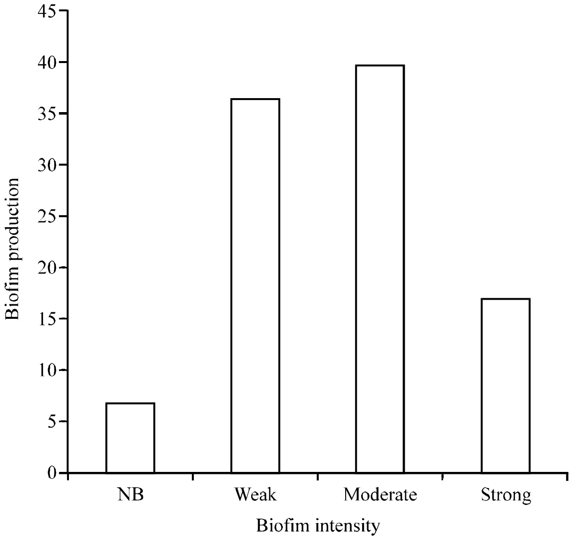

Fig. 1: Percentage of biofilm production among *Proteus* strains isolated in this study, OD: Was measured at 570 nm. NB: No biofilm

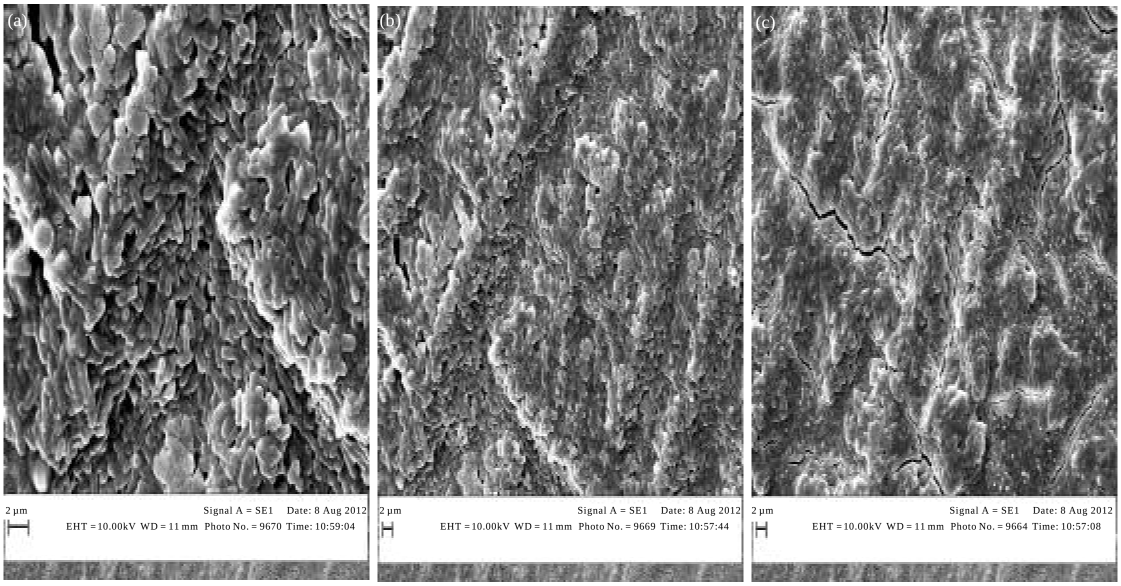

Fig. 2(a-c): Scanning electron micrograph of biofilm production among strong biofilm producing *Proteus* isolates during (a) 8 h, (b) 16 h and (c) 24 h incubation in microtiter plate

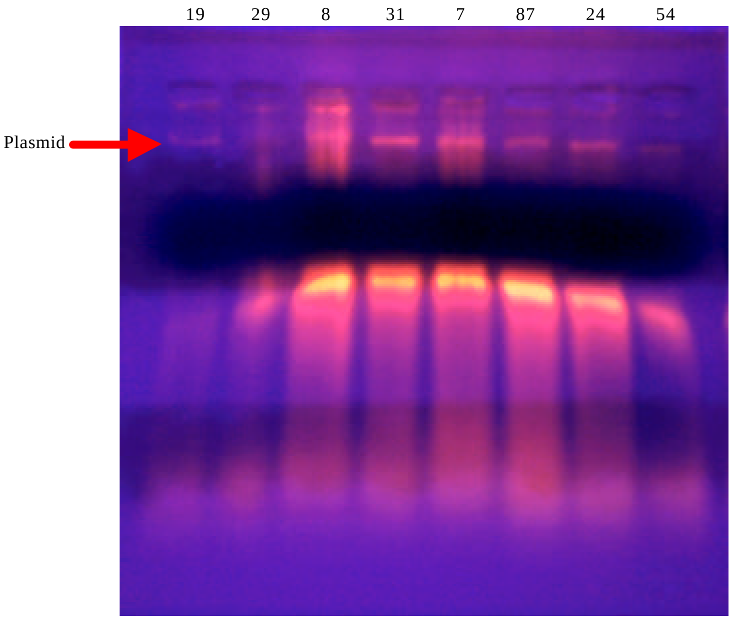

Fig. 3: Agarose gel electrophoresis of plasmid isolated from strongly producing *Proteus* strains isolated in this study. Electrophoresis was conducted for 4 h at 60 volt (35 mA) using 500 mL Tris-Borate-EDTA (TBE) buffer (pH-8.3), Lines 1 to 8 were plasmid bands from isolates No. 19, 29, 8, 31, 7, 87, 24 and 54

result of SEM suggest that as time of incubation in microtiter increased, the quantity of biofilm production was also increased and reached to a maximum within 24 h.

Plasmid isolation: Plasmid isolation from strongly producing biofilm *Proteus* strains revealed that the isolate number 19, 8, 31, 7, 87, 24 and 54 carried similar high molecular weight plasmid. Those strains with no biofilm or weak biofilm did not carry any plasmid. The experiment repeated twice and similar observations were made. Isolate number 29 though exhibited strong biofilm but did not carry any plasmid as shown in Fig. 3.

DISCUSSION

The urinary tract catheters are usually used to remove urine from patients for any reason cannot do normal urination (Trautner and Darouiche, 2004). The problem with catheter is that they are prone to contamination and can easily cause UTI in hospitalized patients using them (Ramsay *et al.*, 1989; Adegbola *et al.*, 1983; Allison *et al.*, 1992). The urinary tract infection is not only important from treatment point of view but also increase considerably the cost of therapy for those patients dependent on them. Majority of the bacteria associated with catheters are resistance to many antibiotics. Previous studies have identified an important association between the administration of inadequate antimicrobial treatment of UTI infection due to biofilm and hospital mortality (Stickler, 2008).

Tambyah *et al.* (1999) reported annually one million of people are infected with contaminated catheter and *Escherichia coli* remains the predominant uropathogen isolated in acute community-acquired uncomplicated infections, followed by *Staphylococcus saprophyticus Klebsiella*, *Enterobacter* and *Proteus* species (Johnson *et al.*, 1993). In one study in Argentina (Aiassa *et al.*, 2010), it was found that the biofilm producing *Proteus* were resistant to ciprofloxacin. Proteus species are playing important role in urinary stone formation by changing the pH of urine due to urease production (Morris and Stickler, 1998).

In this study we found that the *P. mirabilis* were frequently infected UTI people (92.2%) and women contributed the majority of patients (58%). This may be due to close vicinity of urinary tract with vagina. Among biofilms producing strains, only 17.4% could produce strong biofilm and all except one (isolate number 29) carried similar high M.Wt. plasmid. In present study we found that as the time passes the biofilms formation was also increased and it reaches to a plateau after 24 h of incubation. This may be due to increase in production of autoinducer like oxo-dodecaoyl-homoserine lactone (HSL) by this organism. Cox and Hukins (1989) found that the shape of solid substrate also play role in biofilm formation. it was also found that as the length of cathetrization was increased the chance of catheter infection by *Proteus* was also increased.

The susceptibility and antibiotic resistance pattern of Proteus strains isolated from our hospitals revealed that, the majority of the isolates were simultaneously resistance to at least four antibiotics (ceftriaxone, cefotaxime, amikacin and gentamicin) routinely used in our hospitals for treatment of UTI and therefore created problem in therapy of infection caused by this organism. Biofilm producing strains of *P. mirabilis* found to be play Key role in multiple drug resistance phenomenons in this study.

In Iran a few research were carried out in this subject, in one case, Dalal *et al.* (2005) isolated 300 UTI samples isolated from ImamKhomini hospital in Tehran. It was found that *P. mirabilis* was second position among isolates. The results showed amikacin, ciprofloxacin and nalidixic acid were most efficient antibiotics.

Further research must be performed regarding cloning and sequencing of biofilm genes in this organism. Expression of the genes involved in biofilm production among *Proteus* species and how to overcome the biofilm formation.

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