



Full Length Research Paper

Extended spectrum multi-drug resistance versus pathogenic factors- swarming, proteases, and urease- of *Proteus* species

Aktar Uzzaman Chouduri*, Abdul Wadud, Anwar UI Islam

Department of Pharmacy, University of Rajshahi, Rajshahi-6205, Bangladesh

*Corresponding Author Email: auchow5@yahoo.com; Cell Phone: +88-01712792350; Phone: +88-0721-711110 (Office)

ABSTRACT

Multi-drug resistance (MDR) in isolates of *P. vulgaris* (*Pv*), *P. mirabilis* (*Pm*), *P. hauseri* (*Ph*) and *P. penneri* (*Pp*) was determined in our previous study. Here pathogenic features of isolates were investigated. Strong swarming motility was found in isolate 91₂(*Pm*) although MDR had no effect on swarming. Inter-species swarming patterns in *Proteus* were different regardless of MDR-spectrum. *Pv*, *Pm* showed bull's eye swarming pattern and *Pp*, *Ph* featureless mat. Isolates' variable onset times for urea hydrolysis were a consequence of urease producing abilities but not the activities of ureases. Possibly urease production is correlated to the cellular response under urea-induced media. In contrast, total time required to consume a constant amount of urea was identical to intra-species *Proteus* isolates. *Ph* requires 3h, *Pm* 5h, *Pp* 6-7h and *Pv* 9h that can be used as research tool for identification of *Proteus* species. MDR had no effects on the rate of cell proliferation. *Ph* is found as more opportunistic pathogen than others. Strong proteolysis by proteases of isolate 91₂ (*Pm*) was a hyperactive effect of ZapA or other unknown proteases. Thus, 91₂ (*Pm*) marked as most pathogenic *Proteus* strain regarding swarming and proteases. Further qualitative and quantitative analyses of proteases in 91₂ (*Pm*) remain to be elucidated.

Keywords: *Proteus* species, Swarming motility, Urease, Extracellular proteases.

INTRODUCTION

Proteus species are widely distributed in nature. They can be found in polluted water, soil and manure, where they play an important role in decomposing organic matter of animal origin. *Proteus* is opportunistic uropathogenic bacteria which is responsible for asymptomatic infections and persistent recurrences of UTIs. Uropathogenic *Proteus* strains may manifest resistance to several antimicrobials including extended-spectrum cephalosporins, fluoroquinolones, and aminoglycosides (Pagani *et al.*, 2002; Khan and Musharraf, 2004; Wadud and Chouduri, 2013). The increasing evidence of drug resistance in *Proteus* has become a serious problem in effective antibiotic administration. Moreover, many articles have already been reported emphasizing the species *Proteus mirabilis* but little attention has been paid to other species of *Proteus* and they are being overlooked particularly on

their pathogenicity. In our previous study (Wadud and Chouduri, 2013) we isolated and identified eleven *Proteus* isolates of four species, *Proteus vulgaris* (hereafter termed as *Pv*), *P. mirabilis* (*Pm*), *P. hauseri* (*Ph*) and *P. penneri* (*Pp*), from municipal supplied water where all isolates were categorized as multi-drug resistant by disk diffusion method (CLSI, 2012). The spectrums of drug resistance developed in those isolates were scored based on their MAR (Multiple Antibiotic Resistance) index values. The obtained wide spectrums of drug resistance ranging 0.25 to 0.58 indicated an extensive exposure of isolates to high risk sources of human or animal contamination. We speculated that the wide spectrum antimicrobial resistance developed in *Proteus* isolates might have effect(s) on their pathogenic factor(s).

The genus *Proteus* have developed several pathogenic factors including fimbriae, flagella, urease, amino acid deaminases, proteases, toxins (hemolysins, Pta), tissue matrix proteins, swarming growth and LPS which enable them to colonize, survive and grow in the host organism (Coker *et al.*, 2000; Rozalski *et al.*, 2012). Moreover, biofilm formation by *Proteus* species particularly on the surface of urinary catheters is currently creating the serious consequences for patients (Rozalski *et al.*, 2012). The swarming motility contributes the rapid multicellular movement of bacteria across the surface powered by rotating flagella. Thus, here an unresolved question appears as whether the multidrug-resistance (MDR) of *Proteus* has any effect(s) on the enforcement of flagella. A recent work on swarming motility showed that most of the strains among 72 isolates of *Pp* expressed strong to moderate swarming motility and about all strains among 30 isolates of *Pv* expressed strong swarming motility (Kwil *et al.*, 2013). A large group of *Proteus* strains in the same report expressed very weak swarming motility. It is, therefore, unclear that the swarming strengths of *Proteus* over the surface are their species-specific or MDR-dependent consequences.

Another pathogenic factor of *Proteus*, the enzyme urease, catalyzes urea into ammonia and carbon dioxide. This reaction provides nitrogenous nutrition for bacteria and causes the formation of crystalline biofilms that block catheters and form stones in kidney and urinary bladder (Griffith *et al.*, 1976; Mobley *et al.*, 1987; Jones *et al.*, 1990; Mobley and Belas, 1995). Urease production which is specifically induced by urea (Nicholson *et al.*, 1991) appears to be one of the reasons why *Proteus* infection causes more severe histological damage than *Escherichia coli* infection (Johnson *et al.*, 1993; Salyers and Whitt, 1994; Mobley, 1996). The wide spectrum of MDR developed in *Proteus* isolates in our previous study led us to investigate whether urea-induced urease production is affected by the MDR development. Therefore, the objective of this study is to determine the effects of MDR on three selective pathogenic factors of *Proteus*, swarming phenomenon, urease production and proteases, as well as the possible relationships between these pathogenic factors and MDR developed in isolates.

MATERIALS AND METHODS

Bacterial strains and chemicals

Eleven *Proteus* strains of four species (*Pv*, *Pm*, *Ph* and *Pp*) were isolated and identified from 100 samples of municipal tap water as reported in our previous study (Wadud and Chouduri, 2013). All chemicals used in this study were purchased from Sigma-Aldrich, Difco, Thermo Scientific, Oxoid unless it is mentioned otherwise.

Swarming motility test

Bacterial strains were grown overnight in 10 ml of LB broth medium (1% Tryptone, 0.5% Yeast extract, and 0.5% NaCl) at 37°C with shaking (200 rpm). Then 5 µl of fresh cultures was spotted at the center of LB agar plates (LB medium containing 1.5% agar) previously dried to remove drops of water from the surface of the agar medium as described elsewhere (Wilkerson and Niederhoffer, 1995; Kwil *et al.*, 2013) and incubated at 37°C for 44 h. After that the swarm zones were measured in millimeter at three different directions of diameter.

Test for urea hydrolysis

10 ml of Christensen's broth (0.1% peptone, 0.1% glucose, 0.5% NaCl, 0.2% KH₂PO₄, 2% urea and 0.0012% phenol red) adjusted to pH 6 was inoculated with 20 µl of overnight grown bacterial suspension adjusted to OD_{650nm} at 0.5 (~10⁹ CFU/ml) in a graduated culture tube and left it at room temperature (30°C) undisturbed. Urea hydrolysis by the enzyme urease produced by cells was started at the bottom towards the top of the culture tube. The time-dependent change of color of culture broth from yellow to red was visualized and noted as percent based on the graduation mark on the tube (Table 2).

Proteolytic activity measurement

Assay was conducted by casein hydrolysis on nutrient agar plates containing 1.5% of casein. The casein agar plates were pinched and whirled with a sterile metal tube to make wells on them. 20 µl of bacterial cell suspension adjusted to OD_{650nm} 0.5 (~10⁹ CFU/ml) or 20 µl of culture supernatant adjusted to 0.5 µg/µl protein concentration (Bradford, 1976) using BSA as standard was added in each well and incubated overnight at 37°C. Caseinolysis occurred by the secreted proteases of cells was visualized by the formation of a clear zone around wells after precipitation with 1 M HCl solution (Marasabessy *et al.*, 2011).

MAR index calculation

The multiple antibiotic resistance (MAR) index for each isolate was calculated as $MAR = a/b$, where 'a' represents the number of antibiotics the isolate was resistant to and 'b' represents the total number of antibiotics the isolate was tested against (Krumperman, 1985; Wallace *et al.*, 2013).

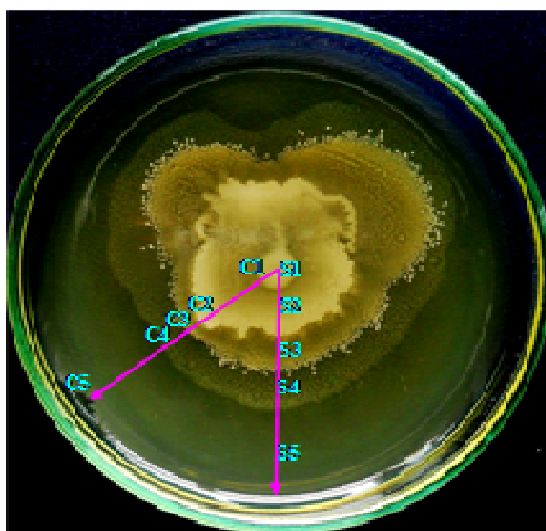


Figure 1. Swarming motility of *Pm* on LB-agar plate. A single colony of isolate 91₂ was spotted at the center of plate and incubated at 37°C for 24 h. Five swarming and consolidation phases are designated as S1, S2, S3, S4, S5 and C1, C2, C3, C4, C5, respectively.

Table 1. Time dependent swarming motility.

| Isolates (Spp.) | Diameter of perimetric swarming phase (mm) | | | | Ranks | Remarks |
|-------------------------------|--------------------------------------------|--------------|--------------|--------------|-------|------------------------------------------------------------------------------------------------------------------------|
| | 10 h | 20 h | 25 h | 44 h | | |
| 11(<i>Pv</i>) | 21.88 ± 0.28 | 28.33 ± 0.58 | 36.33 ± 1.15 | 55.00 ± 1.00 | 5 | Top three scorers were 91 ₂ (<i>Pm</i>), 66 ₂ (<i>Pp</i>) and 91 ₁ (<i>Pm</i>). |
| 91 ₁ (<i>Pm</i>) | 32.46 ± 0.22 | 44.33 ± 2.31 | 49.67 ± 3.06 | 62.67 ± 1.53 | 3 | |
| 91 ₂ (<i>Pm</i>) | 38.20 ± 0.06 | 51.67 ± 4.04 | 57.67 ± 3.51 | 70.67 ± 0.58 | 1 | |
| 66 ₁ (<i>Pp</i>) | 21.02 ± 0.78 | 29.67 ± 0.58 | 35.67 ± 3.06 | 52.67 ± 0.58 | 6 | |
| 66 ₂ (<i>Ph</i>) | 36.98 ± 0.80 | 51.00 ± 3.00 | 54.00 ± 2.67 | 64.67 ± 2.08 | 2 | |
| 66 ₃ (<i>Pp</i>) | 20.22 ± 1.08 | 27.98 ± 1.02 | 34.62 ± 0.32 | 51.37 ± 0.94 | 7 | |
| 66 ₄ (<i>Pp</i>) | 18.62 ± 0.92 | 26.88 ± 0.32 | 32.98 ± 0.86 | 51.00 ± 0.78 | 8 | |
| 66 ₅ (<i>Pp</i>) | 24.02 ± 0.22 | 35.00 ± 1.00 | 38.67 ± 0.58 | 57.00 ± 1.00 | 4 | |
| 66 ₆ (<i>Pp</i>) | 15.09 ± 0.12 | 19.86 ± 0.98 | 27.68 ± 1.04 | 48.50 ± 0.54 | 9 | |
| 66 ₇ (<i>Pp</i>) | 10.11 ± 0.54 | 14.00 ± 1.68 | 25.22 ± 0.78 | 47.02 ± 0.66 | 11 | |
| 66 ₈ (<i>Pp</i>) | 12.12 ± 0.66 | 17.23 ± 0.28 | 26.32 ± 0.56 | 47.78 ± 0.40 | 10 | |

Inoculums were placed at the center of LB agar plates (90 mm diameter) and allowed to swarm at 37°C over time. Values were the means ± SD of at least three individual diameters measured in different directions. Isolates were ranked based on their swarming motility.

RESULTS

Swarming motility

The swarming motility connected with the cell surface components of bacteria is an important virulence factor in cases of UTIs especially for *Proteus* gained through the ascending route. Many questions are still unresolved on the swarming motility of *Proteus* species. Among them one question appeared in our previous study is that "Is there any relationship between MDR developed in *Proteus* and their relevant swarming motility"? To search possible answers we aimed to know whether MDR development in *Proteus* had any effect on the swarming

motility. To date, *Pm* is thought to be a strong swarmer cell among *Proteus* species. A single colony of *Pm* (isolate 91₂) was spotted onto the center of LB agar plate (Figure 1) which showed a distinct swarming growth. The swarming and consolidation phases represented by S and C respectively were appeared clearly as shown in the figure. Then eleven *Proteus* isolates of four species were inoculated at the center of LB-agar plates as described in materials and methods, and allowed to grow at 37°C. Based on isolates' competitive swarming growth for 44 hours they were ranked by measuring diameter of peripheral swarming raft (Table 1). The best scorer cells were 91₂(*Pm*) followed by 66₂(*Ph*) and 91₁(*Pm*) whereas their rankings in MAR index values were 3rd, 4th and 1st,

Table 2. Determination of the ability of isolates to hydrolyze urea.

| Iso | Pr. spp | MAR index | Incubation time (hr) | | | | | | | | | | | | | | | | | |
|-----------------|-----------|-----------|-------------------------------------|---|---|----|----|----|----|-----|-----|----|-----|-----|----|----|----|-----|-----|-----|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| | | | Apparent urea hydrolysis (%) | | | | | | | | | | | | | | | | | |
| 11 | <i>Pv</i> | 0.58 | | | | | | | | 5 | 10 | 20 | 40 | 60 | 70 | 80 | 90 | 100 | | |
| 91 ₁ | <i>Pm</i> | 0.58 | | | 5 | 10 | 20 | 50 | 80 | 100 | | | | | | | | | | |
| 91 ₂ | <i>Pm</i> | 0.42 | | | | | | 5 | 10 | 20 | 30 | 80 | 100 | | | | | | | |
| 66 ₁ | <i>Pp</i> | 0.50 | | | | | | | | | | 5 | 10 | 30 | 50 | 70 | 80 | 90 | 100 | |
| 66 ₂ | <i>Ph</i> | 0.33 | | | | | | 5 | 20 | 70 | 100 | | | | | | | | | |
| 66 ₃ | <i>Pp</i> | 0.42 | | | | | | | | | | 5 | 10 | 30 | 50 | 70 | 80 | 90 | 100 | |
| 66 ₄ | <i>Pp</i> | 0.50 | | | | | | | | | | | | 5 | 10 | 30 | 50 | 70 | 90 | 100 |
| 66 ₅ | <i>Pp</i> | 0.25 | | | | | | | | | | | | 5 | 10 | 40 | 60 | 70 | 80 | 100 |
| 66 ₆ | <i>Pp</i> | 0.42 | | | | | | | | | | | 5 | 10 | 40 | 60 | 70 | 80 | 100 | |
| 66 ₇ | <i>Pp</i> | 0.50 | | | 5 | 10 | 20 | 30 | 40 | 80 | 100 | | | | | | | | | |
| 66 ₈ | <i>Pp</i> | 0.42 | | | | | | 5 | 10 | 30 | 50 | 70 | 90 | 100 | | | | | | |

Each row implicates an apparent urease production by the respective isolates with time. The isolates were ranked based on the time of onset of urea hydrolysis. Ranks were- 91₁(*Pm*) > 66₂(*Ph*), 66₇(*Pp*) > 91₂(*Pm*) > 66₃(*Pp*) > 11(*Pv*), 66₃(*Pp*), 66₆(*Pp*) > 66₁(*Pp*), 66₄(*Pp*), 66₅(*Pp*).

Table 3. Inter- and intra-species ranking of isolates based on their pathogenic factors

| Isolates (Spp.) | MAR | Ranks on MAR | Ranks on Cas.Hyd | Ranks on Gel.Liq | Ranks on Ure.Hyd | Ranks on SM | Remarks |
|-------------------------------|------|--------------|------------------|------------------|------------------|-------------|--------------------------------------------|
| 11(<i>Pv</i>) | 0.58 | 1 | 3 | 1 | 5 | 5 | Extended spectrum-MDR had no effect on SM. |
| 91 ₁ (<i>Pm</i>) | 0.58 | 1 | 5 | 3 | 1 | 3 | |
| 91 ₂ (<i>Pm</i>) | 0.42 | 3 | 1 | 2 | 3 | 1 | |
| 66 ₁ (<i>Pp</i>) | 0.50 | 2 | 2 | 1 | 6 | 6 | |
| 66 ₂ (<i>Ph</i>) | 0.33 | 4 | 4 | 5 | 2 | 2 | |
| 66 ₃ (<i>Pp</i>) | 0.42 | 3 | 2 | 3 | 5 | 7 | |
| 66 ₄ (<i>Pp</i>) | 0.50 | 2 | 2 | 2 | 6 | 8 | |
| 66 ₅ (<i>Pp</i>) | 0.25 | 5 | 3 | 6 | 6 | 4 | |
| 66 ₆ (<i>Pp</i>) | 0.42 | 3 | 6 | 4 | 5 | 9 | |
| 66 ₇ (<i>Pp</i>) | 0.50 | 2 | 4 | 2 | 2 | 11 | |
| 66 ₈ (<i>Pp</i>) | 0.42 | 3 | 6 | 3 | 4 | 10 | |

MDR- Multi-drug resistance, MAR- Multiple antibiotic resistance index, Cas.Hyd- Casein hydrolysis, Gel.Liq- Gelatin liquefaction, SM- Swarming motility

respectively (Table 3). Another isolate 11(*Pv*) bearing highest MAR index value had secured 5th rank in its swarming motility. Otherwise, within the same *Proteus* species (*Pp*), 66₁, 66₄ and 66₇ bearing the same MAR index values (0.50), the isolates' rankings were essentially not even close in their swarming motility (Table 1). Moreover, isolate 91₂ (*Pm*) having the low value of MAR index showed strong swarming motility. The results, therefore, indicated that the spectrum of MDR developed in isolates had no effects on their swarming motilities.

Next the differentiation of intra- and inter-species swarming patterns were investigated with selected

isolates bearing highest and lowest MAR index values (Figure 2). Isolates 11 (*Pv*), 91₁ (*Pm*) and 91₂ (*Pm*) showed bull's eye pattern of swarming motility resembling with other reports (Rozalski *et al.*, 2012; McCall *et al.*, 2013). In isolate 91₂(*Pm*), the rate of swarming was high and swarming rafts were concentric. A swarming pattern of featureless thick mat with rhizoidal center was found in *Ph*, isolate 66₂ (Figure 2B). In isolates 66₁ (*Pp*) and 66₅ (*Pp*), a rhizoidal center surrounded by a narrow ring of clear zone was found (Figure 2C, D). Distinguishable differences of inter-species swarming pattern were found but intra-species swarming patterns were identical regardless of the values of MAR index.

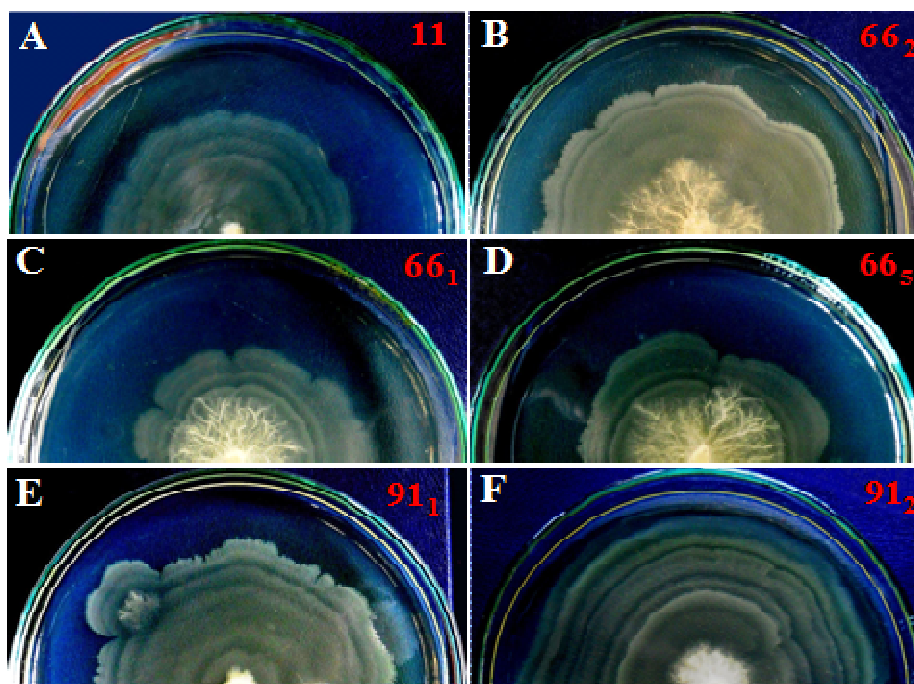


Figure 2. Swarming motility of *Proteus* isolates on LB-agar culture plate. The mean peripheral swarming diameters after 44 hrs incubation at 37°C were A: 55.00, B: 64.67, C: 52.67, D: 57.00, E: 62.67 and F: 70.67 mm for 11 (*Pv*), 66₂ (*Ph*), 66₁ (*Pp*), 66₅ (*Pp*), 91₁ (*Pm*) and 91₂ (*Pm*), respectively. Swarming patterns for 11(*Pv*), 91₁(*Pm*) and 91₂ (*Pm*) were as bull's eye with concentric swarming rafts, for 66₂(*Ph*) as featureless mat with rhizoidal center, for 66₁(*Pp*) and 66₅(*Pp*) as rhizoidal center surrounded by swarming rafts.

Antimicrobial resistance versus urea hydrolysis

Urease, a pathogenic factor of *Proteus*, plays a key role to show the pathogenicity in host cells by urea hydrolysis. Urease also contributes to catheter colonization of *Proteus* species by hydrolyzing urea to NH₃ and CO₂, thereby increasing the pH and facilitating the precipitation of polyvalent ions in urine, leading to the formation of struvite [Mg(NH₄)PO₄] and apatite [Ca₃(PO₄)₂] crystals (Coker *et al.*, 2000; Griffith *et al.*, 1976; Nicholson *et al.*, 1991). Bacterial adherence and colonization typically occurs when the urine pH increases to ~8.2 and crystals deposit on the catheter (Stickler *et al.*, 2006). Here, we aimed to investigate whether MDR developed in isolates had any effect on urease production. We tested the apparent urea hydrolyzing ability of isolates based on the change of color of Christensen's broth medium with time described in materials and methods. The onset of urea hydrolysis was different among the isolates. Two isolates, 91₁ (*Pm*) and 66₇ (*Pp*) started urea hydrolysis after 2 h of incubation and other two isolates, 66₄ (*Pp*) and 66₅ (*Pp*) started after 11 h of incubation. Three possible reasons, (i) the lag phase of growth curve, (ii) the rate of cell proliferation, (iii) the cellular response for urease

production under urea containing medium, might have the relation to the commencement of urea hydrolysis. But first possibility can be ruled out by recently published pH-dependent growth curve of *P. mirabilis* where the lag phase ranged 2.5 to 4 hrs at pH 5 to 10 (Irwin *et al.*, 2013). Thus the second and/or third possibility stands for variable urea hydrolyzing abilities of isolates under urea-induced culture condition. The two species, 11 (*Pv*) and 91₁ (*Pm*), having same MAR index value (0.58) were different in their urea hydrolyzing abilities. The former one was slower than the later one. Otherwise isolates 66₁, 66₄ and 66₇ belonging the species *Pp* and having the same MAR index value (0.50) were also different in their urea hydrolyzing abilities. Therefore, the result indicated that MDR developed in isolates had no effect on apparent urea hydrolyzing abilities but might have connection to the cellular response for urease production.

To confirm whether the onset of urea hydrolysis is related to the cellular response, the standardized isolates (10⁹ CFU/ml) were incubated in Christensen's broth pH 6.0 excluding urea at 37°C. But no growth of cells without urea up to 12 h of incubation indicated that cells were using urea as their nutritional source and the variable times required for the commencement of urea hydrolysis

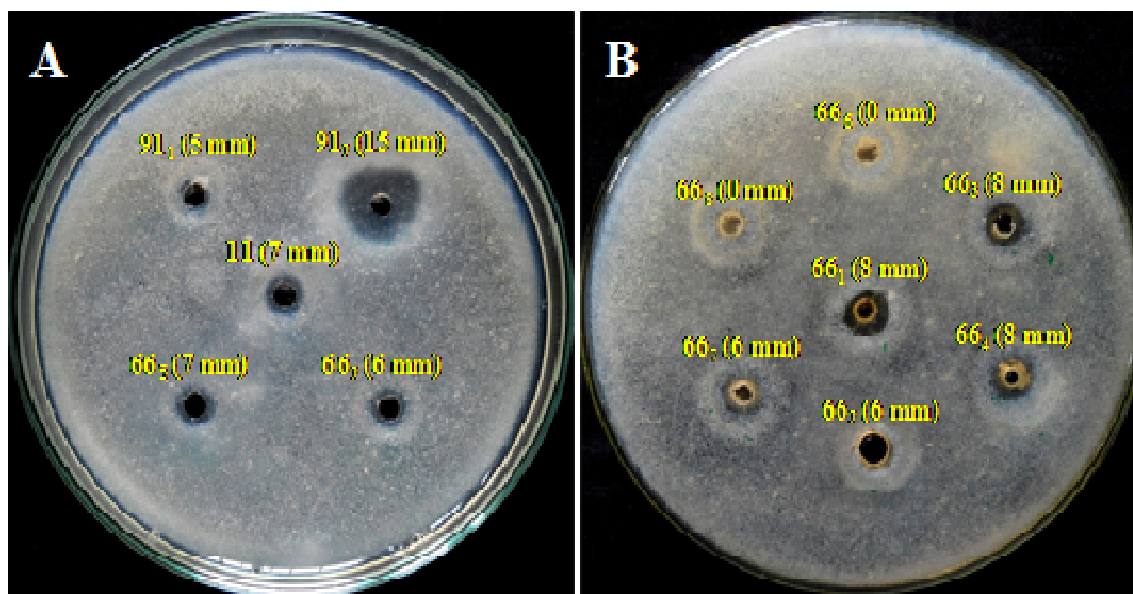


Figure 3. Proteolytic activity of culture supernatant on casein agar plate. Eleven isolates comprising four *Proteus* species categorized as MDR in our previous study (Wadud and Chouduri, 2013) were subjected to a test for caseinolysis. Tested isolates were 11- *Pv*; 91₁, 91₂ - *Pm*; 66₂ - *Ph* and 66₁, 66₃ to 66₈ - *Pp*. Values in parenthesis are diameters (mm) of clear zones produced by secreted proteases of isolates.

by the isolates under urea-induced media had the connection to cellular response for urease production. The same experiment carried out in nutrient broth medium at pH 6.0 showed a consistent rate of cell proliferation for all isolates (data not shown) indicating that the MDR had no effects on the rate of proliferation of cells. The evidence presented here led us to assume that the cellular signals for urease producing or processing abilities by the isolates in urea-induced medium were different which is independent of MDR development. Moreover, although the onset of urea hydrolysis was different the total time required to reach at 100% hydrolysis of urea was the same within the same species, *Pm* and *Pp* (Table 2). For species *Ph* it requires 3 h, for *Pm* 5 h, *Pp* 6-7 h and *Pv* 9 h. Thus, this time can be used as a research/diagnostic tool for rapid identification of *Proteus* species. But further study needs to enforce the finding with more number of isolates for *Ph* and *Pv*.

Proteolysis by extracellular proteases

The bacterial cells and separately culture supernatants were tested for their ability to hydrolyze casein on casein agar plate. The strong zone of caseinolysis (15 mm) was found for isolate 91₂ (*Pm*) indicating that the secreted protease(s) by this isolate was either hyperactive or abundant or rich in substrate specificity (Figure 3A). Following stronger isolates on the basis of proteolytic abilities by their extracellular protease(s) were 66₁ (*Pp*), 66₃ (*Pp*), and 66₄ (*Pp*) exhibiting 8 mm zone of

caseinolysis (Figure 3B). Secreted proteases by the isolates 11 (*Pv*) and 66₅ (*Pp*) were moderately active in caseinolysis but other isolates' performances were relatively weak. Although isolate 11 (*Pv*) and 91₁ (*Pm*) both had the same values (0.58) of MAR index but the abilities of their secreted proteases in caseinolysis were different. Otherwise, within the same species (*Pm*), isolate 91₂ had the lower MAR index value than 91₁ but the proteolytic ability of its extracellular proteases was much higher than that of 91₁. Therefore, the result led us to conclude that the expressed pathogenicity produced by extracellular proteases of isolates is a consequence of their activity, abundance and/or substrate specificity. To date, a metalloprotease secreted by *Proteus mirabilis*, ZapA, is considered to be one of its virulence factors due to its IgA-degrading activity (Wassif *et al.*, 1995; Aneas *et al.*, 2001). Since antibodies and complements are essential elements of the humoral immune system for prevention and control of infections, therefore, the obtained result indicated that protease secretion is a key pathogenic factor to express virulence of *Proteus* species against immune system.

DISCUSSION

Swarming motility is connected with the cell surface components of bacteria, including lipopolysaccharide (LPS). Translocation of swarmer cells is facilitated by an extracellular acidic polysaccharide chain designated as Cmf (colony migration factor), which acts as a lubricant,

reducing surface friction (Gygi *et al.*, 1995). Later this Cmf is characterized which is structurally identical with the long O-antigenic side chains of LPS in *Proteus* (Knirel *et al.*, 2011). The acidic O-antigenic polysaccharides diminish the surface friction during the migration of swarmer cells over the solid surface of the media (Kwil *et al.*, 2013). Our result in this study has created evidence that the MDR spectrum developed in *Proteus* cells had no effect on swarming motility, in other words no effect on the O-antigenic side chains of LPS or the enforcement of rotating flagella. An individual group reported that Zn-starved *Pm* displayed reduced swimming and swarming motilities and produced less *flaA* transcript and flagellin protein, a lubricant secreted by flagella. Swarming motility can be restored by the addition of Zn²⁺ and, to a lesser degree, Fe²⁺ (Nielubowicz *et al.*, 2010). Since no correlation was found between the spectrum of MDR developed in isolates and their swarming motility, therefore, MDR should not have any effect on any other components or factors regarding swarming of *Proteus* including flagellin, Cmf.

The urease is multimeric enzyme with active site containing a di-nickel cluster (Zambelli *et al.*, 2011). Each active site is capped by a 33-residue flap that governs access to and egress from the di-nickel cluster (Pearson *et al.*, 2000). Substrate ingress and product egress from the active site is tightly controlled by the flap (Roberts *et al.*, 2012). Recently Fujihara *et al.* found no difference in urease activity of *Pm* cells grown in acidic broth medium containing urea under different pH conditions (Fujihara *et al.*, 2011). Therefore, our result obtained in urea hydrolyzing abilities (Table 2) by cells of *Proteus* species should be a consequence of urease producing abilities but not the activity of urease. In another report, it has been shown that the dramatic increase in pH as a result of urease activity might have a negative impact on other bacterial species. For instance, *Pm* outcompetes some urease-negative organisms (such as *Enterobacter cloacae*) and some less-potent urease-positive organisms (*M. morgani*, *P. aeruginosa* and *K. pneumoniae*) when co-cultured in a bladder model, even when *Pm* is introduced 72 hours after catheter colonization by the other organism (Macleod and Stickler, 2007). So accelerated urea hydrolysis by *Proteus* cells may exert an inhibitory environment to other microorganism and at the same time the severity of infection. Since our result showed a rapid pH increment for the isolate 66₂ (*Ph*) it can be considered as more opportunistic *Proteus* species than others. Moreover, the total time required to reach at pH enough to change the color of media was indifferent within the same species of *Proteus* (*Ph* - 3 h, *Pm* - 5 h, *Pp* - 6/7 h and *Pv* - 9 h) which can be used as a research or diagnostic tool for rapid identification of *Proteus* species (Table 2). However, the time obtained cannot summarize the generalizing results for whole genus *Proteus* on the basis of examination of eleven strains from tap water, more

strains including clinical isolates need to establish it as a research tool.

Extracellular proteases secreted by *Proteus* cells catalyze human immunoglobulin at its hinge region to combat and survive in host cells. One well known protease to date isolated from *Pm*, ZapA (IgA-degrading metalloprotease), works as a potential virulence factor expressed specifically in swarmer cells, although the *zapA* mutant does not show decreased swarming (Walker *et al.*, 1999). In this study isolate 91₂ (*Pm*) of species *Pm* expressed the highest swarming motility and proteolytic activity in casein agar plate. Therefore, among other isolates studied here 91₂ (*Pm*) could be marked as most pathogenic cell of *Proteus* regarding its ranking on swarming motility and proteases (Table 3). The *zapB* is a recently identified gene necessary for ZapA activity (Ma *et al.*, 2012). Thus the pathogenicity developed in 91₂ (*Pm*) might be a combined effect of ZapA and ZapB proteins or other unidentified proteases secreted by the cells. A qualitative and quantitative analyses of secreted proteases by the cells of 91₂ (*Pm*) remain to be elucidated.

ACKNOWLEDGEMENTS

Authors wish to thank the Department of Pharmacy, University of Rajshahi, Bangladesh for providing laboratory facilities to carry out the entire experiments.

Authors' contribution

AC designed and guided the project, partially conducted experiment and drafted manuscript; AW conducted entire experiment; AU contributed providing suggestions.

Competing interests

None declared

REFERENCES

- Aneas MAF, Portaro FCV, Lebrun I, Juliano L, Palma MS, Fernandes BL (2001). ZapA, a possible virulence factor from *Proteus mirabilis* exhibits broad protease substrate specificity. *Braz J Med Biol Res.* 34: 1397-1403.
- Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72: 248-254.
- Clinical and Laboratory Standard Institute (2012). Performance standards for antimicrobial susceptibility testing, twenty-second informational supplement. 32: 44-36.
- Coker C, Poore CA, Li X, Mobley HLT (2000). Pathogenesis of *Proteus mirabilis* urinary tract infection. *Microbes Infect.* 2: 1497-1505.
- Fujihara M, Obara H, Watanabe Y, Ono HK, Sasaki J, Goryo M, Harasawa R (2011). Acidic environments induce differentiation of *Proteus mirabilis* into swarmer morphotypes. *Microbiol Immunol.* 55: 489-493.

- Griffith DP, Musher DM, Itin C (1976). Urease: The primary cause of infection-induced urinary stones. *Invest Urol.* 13: 346–350.
- Gygi D, Rahman MM, Lai HC, Carlson R, Guard-Petter J, Hughes C (1995). A cell surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis*. *Mol Microbiol.* 17: 1167-1175.
- Irwin NJ, McCoy CP, Carson L (2013). Effect of pH on the in vitro susceptibility of planktonic and biofilm-grown *Proteus mirabilis* to the quinolone antimicrobials. *J Appl Microbiol.* 115(2): 382-389.
- Johnson DE, Russell RG, Lockett CV, Zulty JC, Warren JW, Mobley HLT (1993). Contribution of *Proteus mirabilis* urease to persistence, urolithiasis, acute pyelonephritis in a mouse model of ascending urinary tract infection. *Infect Immun.* 61: 2748–2754.
- Jones BD, Lockett CV, Johnson DE, Warren JW, Mobley HL (1990). Construction of a urease-negative mutant of *Proteus mirabilis*: analysis of virulence in a mouse model of ascending urinary tract infection. *Infect Immun.* 58: 1120-1123.
- Khan AU, Musharraf A (2004). Plasmid-mediated multiple antibiotic resistance in *Proteus mirabilis* isolated from patients with urinary tract infection. *Med Sci Monit.* 10: 598–602.
- Kniriel YA, Perepelov AV, Kondakova A, Senchekova SN, Sidorczyk Z, Rozalski A, Kaca W (2011). Structure and serology of O-antigens as the basis for classification of *Proteus* strains. *Inn Immun.* 17: 70-96.
- Krumperman PH. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods (1985). *Appl Env Microbiol.* 46:165-170.
- Kwil I, Kazmierczak D, Rozalski A (2013). Swarming growth and resistance of *Proteus penneri* and *Proteus vulgaris* strains to normal human serum. *Adv Clin Exp Med.* 22: 165–175.
- Ma Q, Fonseca A, Liu W, Fields AT, Pimsler ML, Spindola AF, Tarone AM, Crippen TL, Tomberlin JK, Wood TK (2012). *Proteus mirabilis* interkingdom swarming signals attract blow flies. *The ISME Journal.* 6: 1356–1366.
- Macleod SM and Stickler DJ (2007). Species interactions in mixed-community crystalline biofilms on urinary catheters. *J Med Microbiol.* 56: 1549–1557.
- Marasabessy A, Moeis MR, Sanders JPM, Weusthuis RA (2011). Enhancing *Jatropha* oil extraction yield from the kernels assisted by a xylan-degrading bacterium to preserve protein structure. *Appl Microbiol Biotechnol.* 90: 2027–2036.
- McCall J, Hidalgo G, Asadishad B, Tufenkji N (2013). Cranberry impairs selected behaviors essential for virulence in *Proteus mirabilis* HI4320. *Can J Microbiol.* 59: 430-436.
- Mobley HLT (1996). Virulence of *Proteus mirabilis*. In: *Urinary tract infection: molecular pathogenesis and clinical management*. Mobley HLT, Warren JW Editors. Washington DC, ASM Press, pp. 245–271.
- Mobley HLT, Belas R (1995). Swarming and pathogenicity of *Proteus mirabilis* in the urinary tract. *Trends Microbiol.* 3: 280–284.
- Mobley HLT, Warren JW (1987). Urease-positive bacteriuria and obstruction of long-term urinary catheters. *J Clin Microbiol.* 25: 2216–2217.
- Nicholson EB, Concaugh EA, Mobley HLT (1991). *Proteus mirabilis* urease: use of a UreA–LacZ fusion demonstrates that induction is highly specific for urea. *Infect Immun.* 59: 3360–3365.
- Nielubowicz GR, Smith SN, Mobley HLT (2010). Zinc uptake contributes to motility and provides a competitive advantage to *Proteus mirabilis* during experimental urinary tract infection. *Infect Immun.* 78: 2823–2833.
- Pagani L, Migliavacca R, Pallecchi L, Matti C, Giacobone E, Amicosante G, Romero E (2002). Emerging extended-spectrum beta-lactamases in *Proteus mirabilis*. *J Clin Microbiol.* 40: 1549–1552.
- Pearson MA, Park IS, Schaller RA, Michel LO, Karplus PA, Hausinger RP (2000). Kinetic and structural characterization of urease active site variants. *Biochemistry.* 39: 8575-8584.
- Roberts BP, Miller BR, Roitberg AE, Merz KM (2012). Wide-open flaps are key to urease activity. *J Am Chem Soc.* 134: 9934–9937.
- Rozalski A, Torzewska A, Moryl M, Kwil I, Maszewska A, Ostrowska K, Drzewiecka D, Zablotni A, Palusiak A, Siwinska M, Stacek P (2012). *Proteus* sp. - an opportunistic bacterial pathogen - classification, swarming growth, clinical significance and virulence factors. *Folia Biologica et Oecologica.* 8: 1–17.
- Salyers AA, Whitt DD (1994). Host defenses against bacterial pathogens, defenses of body surfaces. In: Salyers AA, Whitt DD Editors. *Bacterial pathogenesis: a molecular approach*. 2nd ed. Washington DC, ASM Press, pp. 3–15.
- Stickler DJ, Lear JC, Morris NS, Macleod SM, Downer A, Cadd DH, Feast WJ (2006). Observations on the adherence of *Proteus mirabilis* onto polymer surfaces. *J Appl Microbiol.* 100: 1028–1033.
- Wadud A, Chouduri AU (2013). Microbial safety assessment of municipal water and incidence of multi-drug resistant *Proteus* isolates in Rajshahi, Bangladesh. *Curr Res Microbiol Biotechnol.* 1: 189-195.
- Walker KE, Moghaddame-Jafari S, Lockett CV, Johnson D, Belas R (1999). ZapA, the IgA-degrading metalloprotease of *Proteus mirabilis*, is a virulence factor expressed specifically in swarmer cells. *Mol Microbiol.* 32: 825–836.
- Wallace CC, Yund PO, Ford TE, Matassa KA, Bass AL (2013). Increase in antimicrobial resistance in bacteria isolated from stranded marine mammals of the Northwest Atlantic. *Ecohealth.* 10: 201-210.
- Wassif C, Cheek D, Belas R (1995). Molecular analysis of a metalloprotease from *Proteus mirabilis*. *J Bacteriol.* 177: 5790–5798.
- Wilkerson ML and Niederhoffer C (1995). Swarming characteristics of *Proteus mirabilis* under anaerobic and aerobic conditions. *Anaerobe.* 1: 345-350.
- Zambelli B, Musiani F, Benini S, Ciurli S (2011). Chemistry of Ni²⁺ in urease: Sensing, trafficking, catalysis. *Acc Chem Res.* 44: 520-530.

How to cite this article: Chouduri A.U., Wadud A., Ul Islam A. (2014). Extended spectrum multi-drug resistance versus pathogenic factors- swarming, proteases, and urease- of *Proteus* species. *Int. Res. J. Microbiol.* 5(1):8-15