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*Full Length Research Paper*

# Strong cephalosporin resistant uropathogen, *Proteus mirabilis*, in urban tap water harbors a risk to public health, Bangladesh

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**Eleven *Proteus* strains belonging four species were isolated in our previous study aimed for microbial quality assessment of urban supplied water. The obtained HPC ranged from $1\times10^3$ to $296\times10^3$ CFU/ml and TCC from 10 to 890 CFU/ml which exceeded WHO, ISI and USEPA minimal limits that indicated the presence of pathogens of different genus and species in samples. Elevated TCC was an indicator of pathogens from fecal origin. Two isolates, designated as Pm1 and Pm2, were identified as *Proteus mirabilis* that were strongly resistant to cephalosporins of ceftazidime, cefixime, ceftriaxone indicating the exposure of isolates to antimicrobials from human or animal sources in nature and the inhibitory pathway of cephalosporins were protected in these isolates. The spectrum of resistance against twelve tested antibiotics in Pm1 was higher (0.58) than that of Pm2 (0.42) based on their MAR index. Alternatively backdated drugs- penicillin, amoxycillin, ampicillin- showed strong inhibition of all isolates including Pm. Now-a-days, the extensive use of cephalosporins offered the pathogens to be resistant against them but the effectiveness of backdated drugs revealed the revival of antibiotics that still can be used for infection control. The swarming motility and cell proliferation of Pm isolates were independent of the spectrum of resistance.**

**Keywords:** *Proteus mirabilis*, strong cephalosporin resistance, amoxycillin, cell proliferation.

## INTRODUCTION

Drinking water must be free from components which may adversely affect the human health. Such components include minerals, organic substances and disease causing microorganisms. A large portion of the population in developing countries suffers from health problems associated with either lack of drinking water or due to the presence of microbiological contaminants in water (Van Leeuwen, 2000). Poor water quality is responsible for the death of an estimated 5 million children in the developing countries (Holgate, 2000). The problem is rapidly aggravated by increasing population resulting in poor water-quality management (Huang and Xia, 2001).

Safety and quality of drinking water is always an important public health concern (Hrudey and Hrudey, 2007 and Reynolds et al., 2008) as contamination of potable water has been frequently found associated with transmission of diseases causing serious illness and

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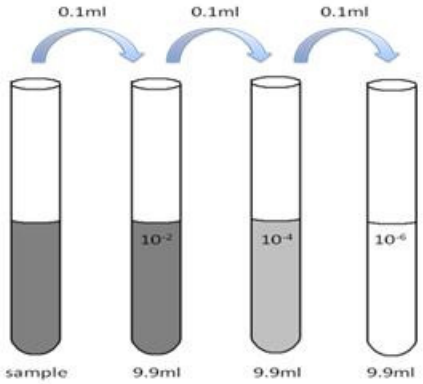


**Figure 1.** Schematic diagram of serial dilution technique. 10 ml each of the tap water samples were aseptically and serially diluted in distilled water. 200 µl of diluted samples were inoculated on nutrient agar plate for heterotrophic plate count.

mortality throughout the world (Marshall et al., 2006; Jones et al., 2007 and O'Reilly et al., 2007). Although poor sanitation and food sources are integral to enteric pathogen exposure, drinking water is a major source of microbial pathogens especially in developing countries (Ashbolt, 2004). Among waterborne diseases of bacterial origin typhoid fever, bacillary dysentery and diarrhea are common in Bangladesh (Parveen et al., 2008 and Begum et al., 1999). Despite of the availability and promotion of the use of safe water sources, water-related diseases remain an important cause of mortality and morbidity in Bangladesh (Mitra, 1992). In Asian and African countries children are most affected by microbial diseases transmitted through water (Seas et al., 2000). The main bacterial diseases transmitted through drinking water are cholera, gastroenteritis, typhoid fever, Salmonellosis, Bacillary dysentery or Shigellosis.

Many studies have illustrated the usefulness of indicator bacteria in predicting the presence of pathogens in water (Wilkes et al., 2009). Coliforms are generally accepted as an indicator for sewage pollution. The tap water samples of studied area, Rajshahi city, Bangladesh, have been shown as sewage polluted in our previous study (Wadud and Chouduri, 2013) and here we aimed to assess the heterotrophic plate count (HPC) and total coliform count (TCC) of tap water samples over the city. Eleven Proteus isolates comprising four species have been identified (Wadud and Chouduri, 2013) where Proteus mirabilis (Pm) is thought to be the most pathogenic among other *Proteus* species. Pm is an opportunistic pathogen, is especially problematic as a urinary tract pathogen in catheterized patients, those with spinal cord injury, or those with anatomical abnormality of the urinary tract (Coker et al., 2000). This urease-positive bacterium causes an increase in urinary pH and the production of kidney and bladder stones (Griffith et al., 1976 and Li et al., 2002). In addition, urinary catheters become encrusted and even blocked during Pm infections (Mobley and Warren, 1987). Numerous studies have been conducted in an effort to understand Pm about its pathogenicity, role of pathogenic factors, drug resistance, swarms, antimicrobial susceptibility and so on, yet despite some advantages, much remains unknown. Therefore, this research was targeted at determining the HPC and TCC as indicators of water quality, the current drug resistance profile of Pm, and the possible relationship of drug-resistance on swarming and cell proliferation of Pm.

## MATERIALS AND METHODS

### *Sample collection*

Total 100 tap water samples were collected in sterile amber-glass bottles kept in icebox from different points of localities over the Rajshahi City Corporation area. The experimental processes were carried out within 1-8 hours after its collection. For few cases the samples were kept at 4℃ in a refrigerator until use. Distilled water or water for injection was used as negative control.

### *Culture media and antimicrobial disks*

The culture media used in this study were Nutrient broth, Nutrient agar, and Muller-Hilton agar purchased from Hi-Media, India; MacConkey agar from Oxoid, Cambridge, UK; Kligler Iron agar from Sifin, Germany; Peptone water prepared in laboratory. All media were prepared as directed by the manufacturer. For antimicrobial susceptibility test all antibiotic disks were purchased from Oxiod, England and Hi media, India. The doses of antibiotics and disc diameters were in accordance with the standards of World Health Organization.

### *Heterotrophic plate count (HPC)*

Each sample was serially diluted with sterile distilled water following the sequential dilution procedure (Figure 1). 200

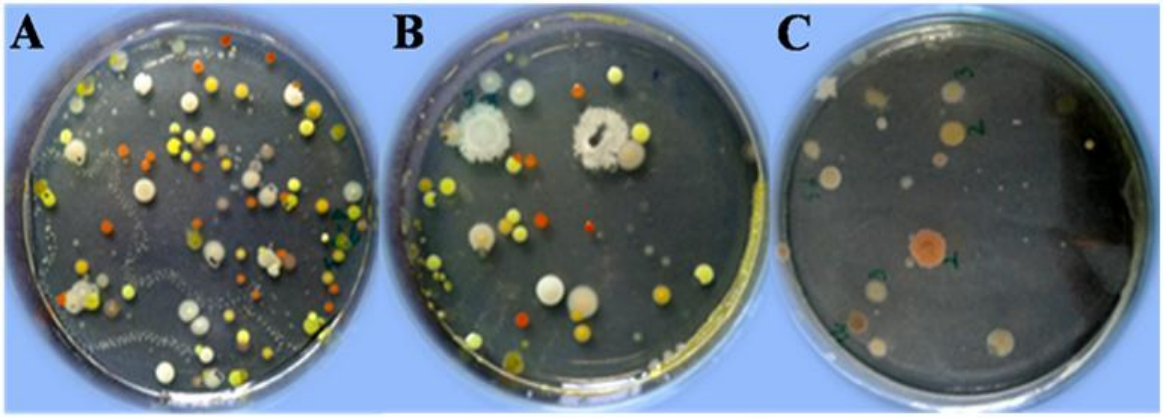


**Figure 2.** Sample (No. 24), was inoculated on nutrient agar plate following the serial dilution technique. Colorful bacterial colonies were formed after 24 h incubation at 37°C. HPC was calculated multiplying the number of CFU with dilution factors. Dilution factors are A: $10^2$, B: $10^4$, C: $10^6$.

µl of each dilution was spread on Nutrient agar plate for heterotrophic plate count (HPC) and MacConkey agar plate for total coliform count (TCC) (Charity et al., 2012). Plates of appropriately diluted samples were incubated overnight at 37°C for HPC and at 45°C for TCC. The HPC and TCC for each sample were obtained as CFU/ml by multiplying the total number of bacterial colony formed on culture plate with the dilution factor. For analysis values were taken as means from at least three individual dilutions.

### *Carbohydrate fermentation*

The test was performed by inoculating a loopful culture of the isolated organisms into the culture tubes containing Peptone Water media with six different basic sugars, glucose, sucrose, lactose, maltose, D-Xylose and mannitol, and incubated for 24 hours at 37ºC. Acid production was indicated by the color change of the indicator methyl red added in media from reddish to yellow. The sugars are added in concentration ranging from 0.5 to 1% (Choudhury, 1999).

### *Indole test*

Testing of indole production is important in the identification of enterobacteria. Most strains of the family Enterobacteriaceae like *E. coli*, *P. vulgaris*, *P. rettgeri*, *M. morganii* and *Providencia* species breakdown the amino acid tryptophan with the release of indole. Tryptone broth medium was used for the test. The indole positive reaction produces a cherry-red ring floating above the culture medium.

### *Antimicrobial susceptibility tests*

The antimicrobial susceptibility profile of isolates was determined using the standard disc diffusion method (Bauer et al., 1966). Standardized inoculums of the overnight grown LB broth cultures were spread on Mueller-Hinton agar plates using sterile swabs. The plates were dried at room temperature for 2 hrs before placing the antibiotic disks at equidistance. The plates were incubated at 37ºC and the diameters of zone of inhibition were measured after 18 hr. The interpretation of zone diameter was followed as recommended in the Clinical Laboratory Standard Institute (CLSI, 2012).

## RESULTS AND DISCUSSION

### *Heterotrophic Bacterial count and public health risk*

All of 100 samples were subjected to HPC analysis for microbiological safety assessment of urban tap water over the city. The obtained HPC ranged from $1\times10^3$ to $296\times10^3$ CFU/ml (Figure 3). For HPC analysis the maximum level of microbial contaminants that is allowed in drinking water is not more than 500 CFU/ml which is enforceable standard by Drinking Water Standards and Advisories 2012. However, the samples tested were found as unhygienic to drink that might produce gastroenteritis, diarrhea, dysentry or other waterborne diseases to users. In most of the samples we found variably colored bacterial colonies formed on culture plate which indicated the presence of numerous pathogenic bacteria of different genus and species (Figure 2) in the sample.

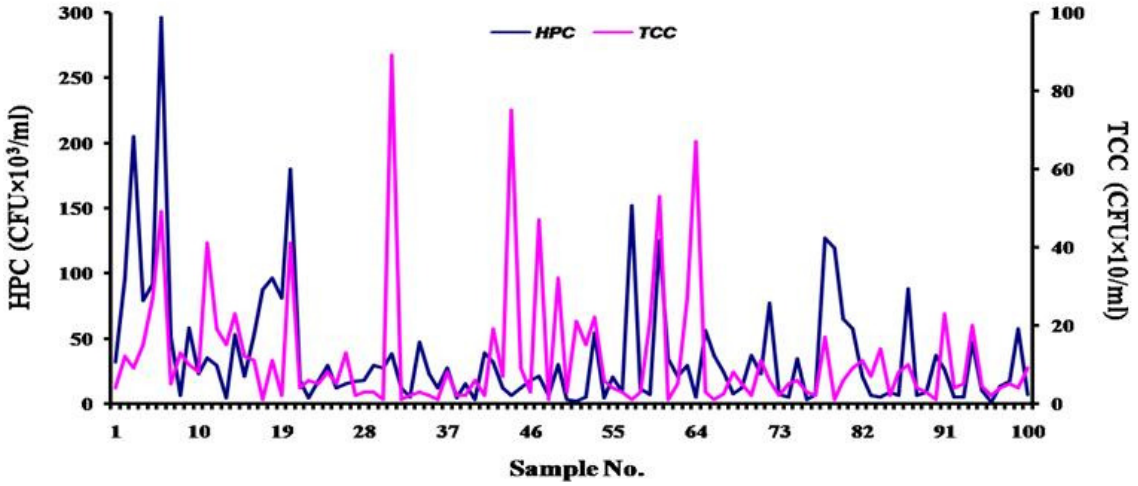


**Figure 3.** Comparative analysis of HPC and TCC. HPC (blue line) ranged from $1\times10^3$ to $296\times10^3$ CFU/ml and TCC (pink line) ranged from 10 to 890 CFU/ml. Both counts were above the minimal limit of WHO standard.

Urban supplied water is generally used in household purposes like drinking, cleaning, cooking. Coliforms are indicator organisms of drinking water that are not normally the causes of serious illness but their presence is used to indicate that other pathogenic organisms of fecal origin may be present. Typical genera of coliforms include *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, Fecal coliform, and *Escherichia*. We determined TCC of each sample in addition to HPC which ranged between 10 to 890 CFU/ml. Elevated TCC was found in samples S31, S44, S64, S60, S6, S47, S11 and S20 having the values 890, 750, 670, 530, 490, 470, 410 and 410 CFU/ml, respectively (Figure 3) and these sampling areas were Senanibas, Mita Studio, Sonadighir more, Ponchoboti, TB hospital quarter, Dashmary, Ranibazar and Sopura, respectively.

The result indicated that pathogenic organisms from fecal origin were present in sampled water of these areas which reflects the public health risks associated with the consumption of water. The USEPA coliform limit in drinking water is less than 1 CFU/100 ml (Van Leeuwen, 2000). Here all samples exceeded this limit. Moreover, the comparative survival of coliform bacteria has a half-life of 17 hrs which is greater than that of other pathogenic bacteria (*S. typhi* 6 h, *V. cholerae* 7.2 h) (Mitra, 1992). Many roadside restaurants over the studied city use to store tap water for a long time and are extensively using this water as drinking purpose to the customers. Since the half life of coliform is high, thus, the customers need to be alert about the usage of water in restaurants to avoid sufferings from enteric diseases, typhoid fever, pneumonia and other waterborne diseases.

### *Identification of Proteus mirabilis*

The samples collected were aseptically inoculated on culture plates of KIA media following the serial dilution technique (Figure 1) and incubated at 37ºC for 24 hrs. The black centered colonies formed on culture plates were searched which supposed to be the genus Salmonella or Proteus. The suspected colonies were picked up and their morphological characteristics including size, shape, color, pigmentation, microscopic observation were recorded. Eleven *Proteus* isolates were then identified by sequential biochemical screening described in our previous study (Wilkes et al., 2009) based on whether they were positive for nitrate reduction, $H_2S$ gas production, methyl-red and urease reactions, and glucose fermentation; negative for lactose and mannitol fermentation. The two isolates among others, $91_1$ and $91_2$, showed indole negative and urease positive reactions (Figure 4).

These isolates were lactose and mannitol non-fermenter that supposed them to be a species of either Morganella or Proteus (Figure 5). In contrast, sucrose fermentation by the isolates confirmed them as *Proteus* species and further maltose non-fermentation designated the isolates as mirabilis species, i.e. *Proteus mirabilis* (Figure 5).

The isolates $91_1$ (hereafter termed as $Pm_1$) and $91_2$ ($Pm_2$) were then subjected to antimicrobial susceptibility tests with several antibiotics as mentioned in material section.

### *Update of antimicrobial resistance profile*

Antimicrobial susceptibility profile of Pm isolates was

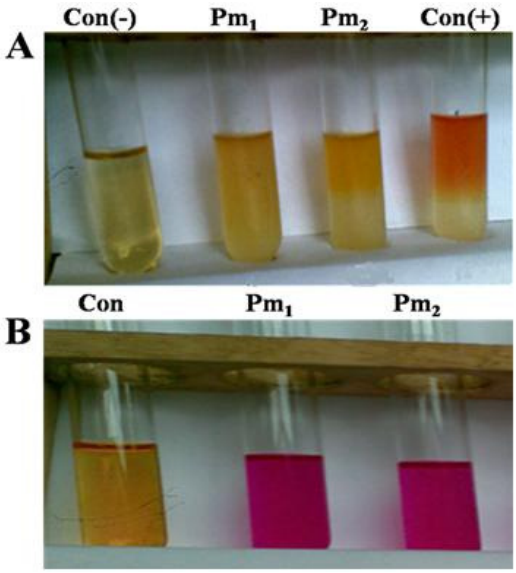


**Figure 4.** Biochemical screening for *Proteus mirabilis* (Pm). Isolates $91_1$ and $91_2$ indicated as $Pm_1$ and $Pm_2$ respectively were subjected to biochemical screening of indole test and urea hydrolysis. Both Pm1 and Pm2 showed indole negative (A) and urease positive (B) reactions.

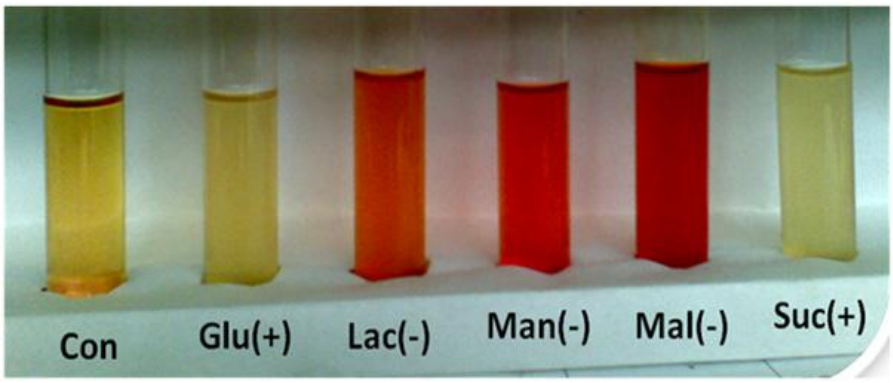


**Figure 5.** Carbohydrate fermentation test. Isolate $91_1$ ($Pm_1$) was allowed to ferment five basic sugars- glucose, lactose, mannitol, maltose, and sucrose- as carbohydrate in peptone water base containing phenol red indicator. Positive reactions for carbohydrate fermentation turned the color of media from reddish to yellow. Con: positive control.

documented against twelve antibiotics belonging six groups by disk diffusion method. Among the antibiotics tested against the isolates, seven were resistant and four others were intermediately resistant to $Pm_1$. In contrast, five antibiotics were resistant and two others were intermediately resistant to $Pm_2$. Here the spectrum of resistance against the tested antibiotics was higher in $Pm_1$ than that of $Pm_2$. Details are described in our previous study (Wadud and Chouduri 2013). Three representative agar plates with zone of inhibition have been shown in figure 6.

Notably two antibiotics, ceftriaxone and ceftazidime, showed no zones of inhibition (Figure 6A) and two others, cephradine and cefixime, showed very small zones of inhibition (Figure 6B) indicating that the inhibition mechanism by cephalosporins are strongly protected in these isolates. Moreover, amoxicillin, ampicillin, penicillin showed strong zone of inhibition (Figure 6C). Therefore, it is interesting that recently updated drugs are generally and preferably used by the physicians to control the infectious diseases but here the isolates derived from natural source were strongly resistant to these updated drugs like third generation cephalosporins- ceftazidime, cefixime, ceftriaxone indicating the exposure of isolates to antimicrobials from human or animal sources in nature. Alternatively backdated drugs such as penicillin, amoxycillin, ampicillin showed strong inhibition against all isolates including Pm. The obtained result led us to conclude that the inhibitory pathway through which the

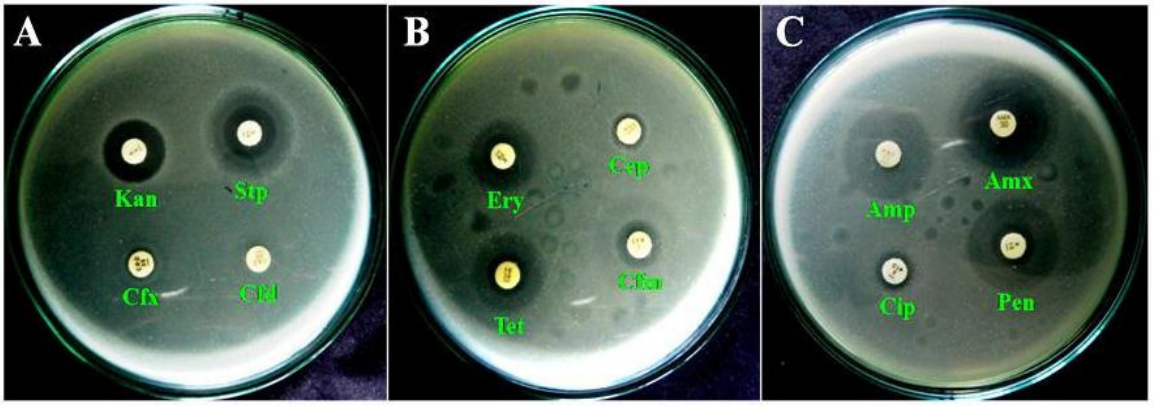


**Figure 6.** Antimicrobial susceptibility tests performed by disk diffusion method. Tested antibiotics are - Kan: Kanamycin, Stp: Streptomycin, Cfx: Ceftriaxone, Cfd: Ceftazidime, Ery: Erythromycin, Cep: Cephradine, Tet: Tetracycline, Cfm: Cefixime, Amp: Ampicillin, Amx: Amoxycillin, Cip: Ciprofloxacin, Pen: Penicillin.

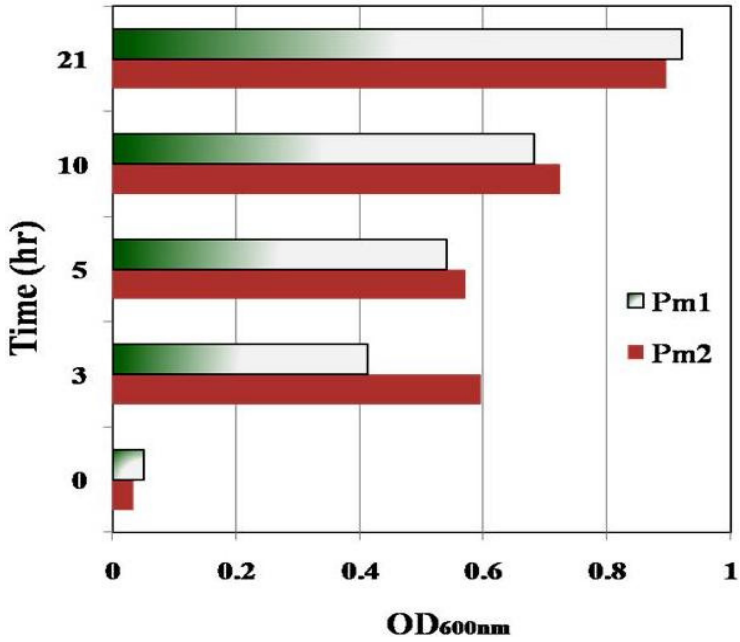


**Figure 7.** Rate of cell proliferation. Freshly prepared standardized cells ($10^9$ CFU/ml) were grown in nutrient broth media adjusted to pH 10 at 37°C over indicated time. Cell proliferations with time were essentially same for both *P. mirabilis* strains, $Pm_1$ (gradient filled bar) and $Pm_2$ (solid filled bar).

updated drugs are acting against the bacterial cells especially *Proteus* species is already protected whereas the inhibitory pathway of backdated drugs remains unprotected. Thus, we assumed that now-a-days extensive use of cephalosporins offered the pathogens to be resistant against them but backdated drugs were effective in contrast that demonstrated the revival of antibiotics for infection control.

### *Antimicrobial resistance and cell proliferation*

The spectrum of antimicrobial resistance of Pm isolates has already been determined in our previous study (Wadud and Chouduri, 2013), by measuring the MAR (Multiple Antibiotic Resistant) index where $Pm_1$ and $Pm_2$ had a MAR index value of 0.58 and 0.42, respectively. Both isolates showed bull's eye pattern of swarming on LB agar plate resembling with other investigators (Reynolds et al., 2008; Seas et al., 2000 and McFeters et al., 1974). $Pm_2$ was strong swarmer than $Pm_1$ and the spectrum of resistance did not have any effect on swarming motility (Unpublished data). Since $Pm_2$ having short spectrum of resistance showed strong swarming motility than $Pm_1$ having broad spectrum of resistance, thus, a question appears as whether broad spectrum resistance has any negative effect

on swarming motility or cell proliferation. We have already proved that the spectrum of resistance has no effect on swarming motility (unpublished data) and since optimal growth for replication of Pm is pH 7 (Jones et al., 2007) here the isolates were allowed to grow at alkaline media (pH 10) which might have effect on cell proliferation. But our result (Figure 7) implicated that the rate of cell proliferation is independent of spectrum of resistance developed in isolates $Pm_1$ and $Pm_2$.

## CONCLUSION

The report has confirmed that urban supplied water in Rajshahi is not free from enteric pathogens and may expose users to various waterborne diseases. Care should be taken for the usage of tap water as it is a reservoir of pathogenic organisms. Strong cephalosporin resistant pathogenic *Proteus mirabilis* can easily be killed by using backdated drugs like penicillins, kanamycin, which is an update to infection control and management.

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## AUTHORS' CONTRIBUTION

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

## COMPETING INTERESTS

None declared

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