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Original Article

Class 1 Integron gene harbouring multidrug resistant *Pseudomonas* isolates from clinical specimen

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Gram-negative *Pseudomonas* spp. particularly *P. aeruginosa* is an important pathogen in hospitalized patients, contributing to the morbidity and mortality due to its multiple resistance mechanisms. The present study was undertaken to determine the characteristics of *Pseudomonas* spp. isolated from pus of patients admitted in a local diagnostic center, Dhaka, Bangladesh. A total of 14 clinical isolates were obtained. Detailed biochemical tests, antibiotic resistance pattern and class 1 integron gene profiling were successfully performed. All isolates (100%) showed resistance against amoxicillin-clavulanate, ampicillin, ciprofloxacin, cephalexin, ceftriaxone, kanamycin, nitrofurantoin and rifampicin. About 35% of the isolates (n=5) showed resistance against Amoxicillin-clavulanate, ampicillin, azithromycin, amikacin, ciprofloxacin, cephalexin, co-trimoxazole, ceftriaxone, imipenem, kanamycin, nitrofurantoin and rifampicin. Only 28.5% isolates (n=4) were sensitive to Imipenem. Plasmid was observed in none of the isolates, whereas 50% of the isolates carried Class 1 Integron gene. The presence of mobile genetic element is an added threat as this enables transfer of resistance genes to sensitive isolates. The findings of this study are significant from a public health viewpoint as therapeutic options become restricted.

Keywords: *Pseudomonas*, Antibiotic Resistance, Class 1 Integron gene, Clinical Sample

Introduction

Antibiotic resistance has been on the rise in the recent years[1,2] (European Centre for Disease Prevention and Control (ECDC), 2017; World Health Organization (WHO), 2014). Developing coutnries are more affected by antibiotic resistance because of the widespread indiscriminate use of antibiotics, use of antibiotics in animal feed, inadequate surveillance and factors associated with individual and national poverty[3,4] .

Pseudomonas spp. particularly *P. aeruginosa* is an opportunistic pathogen that may cause severe invasive diseases in critically ill patients. Strains of *P. aeruginosa* are the cause of several diseases in nosocomial environments, predominantly pneumonia, bacteremia, meningitis, urinary tract infections, as well as skin and soft tissue infections[5-7].

The frequency of infections caused by them is increasing and multidrug-resistant (MDR) strains, resistant to almost all antimicrobials, are emerging in hospitalized patients. Because of its ubiquitous nature, ability to survive in moist environments, and resistance to many antibiotics and antiseptics, *P. aeruginosa* is a common pathogen in hospitals and particularly in intensive care units. It has been reported that 80% of the deaths in ICU patients in Bangladesh is due to multidrug resistant bacteria (The Telegraph, 2019)[8]. Mobile genetic elements such as plasmids, transposons, and integrons play an important role in aggravating antibiotic resistance [9-11]. Class 1 integrons contain exogenous gene cassettes and play an important role in horizontal transfer of antibiotic resistance genes among bacteria [12-16, 17, 18].

Over the years, increases in the rate of antibiotic resistance to *P. aeruginosa*, in particular to â-lactams, aminoglycosides, and fluoroquinolones, have been reported from many parts of the world[19]. The present study was undertaken to investigate antibiotic resistance profile of *P. aeruginosa* isolated from pus of patients admitted in a hospital in Dhaka city.

Materials and Methods

Bacterial Strains

A total of 14 *Pseudomonas aeruginosa* stored in the laboratory (Department of Microbiology, University of Dhaka, Bangladesh) repository were included in the study. These isolates were obtained from pus samples from a local hospital.

Subculture

For the growth of isolated colony, the organisms were subcultured on Nutrient agar plates and incubated at 37°C for 24 hours. After that, one isolated colony was inoculated on Cetrimide Agar (CA) plate. After further incubation of the CA plates at 37°C for 24 hours, cultures of CA plates were transferred to a 1.5 ml Eppendorf

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tube containing LB (Luria Bertani) with 70% glycerol and stored at -20°C.

Morphological and Biochemical test

All the isolates were identified by culture characteristics and Gram staining in the laboratory. Selective media were used to identify them Cetrimide agar plates were used to identify *Pseudomonas aeruginosa* as well as to observe cultural characteristics, where they produced characteristic yellow green color.

Further confirmation of test isolates was done by conventional biochemical tests. Biochemical tests were performed and interpreted according to the methods described in Microbiology Laboratory Manual[20]. The tests included Kligler's Iron Agar (KIA) test, Citrate test, Motility, Indole, Urease (MIU) test, lndole test, catalase test and Oxidase test.

Determination of the Antibiotic Resistance Pattern of the Isolates

Disk diffusion method described by Bauer and Kirby (1969)[21] and recommended by Clinical Laboratory Standard Institute (CLSI*,* 2018)[22] was followed to determine antibiotic resistance pattern. The antibiotic disks used in this study were: Amoxycillin-clavulanate (30µg) Ampicillin (10µg), Amikacin (30µg), Azithromycin (15ìg), Ciprofloxacin (5ìg), Cephalexin (30µg), Cotrimoxazole (sulfamethoxazole 23.75 µg + trimethoprim 1.75 µg), Ceftriaxone (30µg), Imipenem (10µg), Kanamycin (30ìg), Nitrofurantoin (300ig), and Rifampicin (5ìg). The diameter of zone of inhibition for an individual antimicrobial agent was translated into susceptible, intermediate, or resistant categories according to the CLSI guidelines (2018)[22].

Plasmid Profiling

Plasmid DNA was prepared according to the simplified alkaline lysis method of Birnboim and Doly (1979)[23]. Plasmid DNA was separated by horizontal electrophoresis in 0.8% agarose slab gels in a TAE buffer at room temperature at 80 volts for 1.5 hours. The gel was stained with Et-Br for 25 minutes and destained with distilled water for 5 minutes. DNA bands were visualized and photographed using Gel Documentation with UV trans-illuminator.

Integron Profiling

PCR was performed to amplify the Integron genes using a pair of Integron gene-specific primers (*intl*F 5¹-ACATGTGAGGCGACGCACGA-3¹, *intl*R 5¹-ATTTCTGTCCTGGCTGGCGA-3¹)[24]. The PCR was performed in total volume of 50 µl containing 25 µl of PCR master mix (GeneOn, Germany), 2 µl DNA template, 0.5 µl of each primer, 0.25 l of DMSO and 21.75 µl PCR grade water. For completion of PCR, the thermocycler was programmed: Initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C, extension at 68°C for 30 seconds and final extension at 68°C for 10 minutes. The PCR products were resolved by horizontal electrophoresis in 1.2% agarose slab gel. The gel was stained and the DNA bands were visualized as aforementioned.

Results

Cultural and morphological identification P. aeruginosa

Pseudomonas aeruginosa were re-confirmed by observing yellowish green colonies on CA plates with characteristics grape–like odor of aminoacetophenone (Fig. 1).

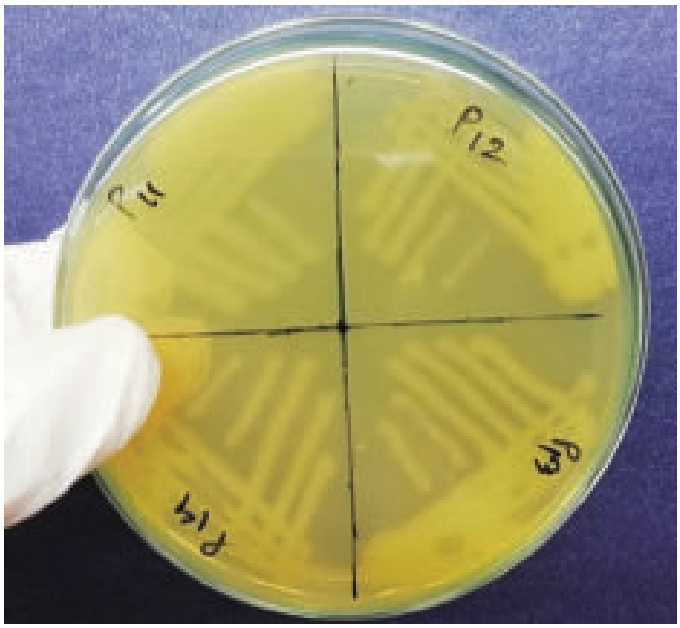

Fig. 1. *Morphological characteristics of Pseudomonas colonies on CA*

Biochemical Characteristics

All isolates conformed to biochemical reactions typical of *Pseudomonas* spp. by producing red slant, red butt, no gas and no H_2S in Kligler Iron Agar. All isolates were Indole negative, utilized Citrate as sole Carbon source and were Oxidase and Catalase positive.

Antibiotic Resistance Pattern

All isolates were resistant to at least 6 of the 13 antibiotics used and were hence MDR. All the isolates were resistant to Ampicillin, Amoxycillin, Cephalexin and Rifampicin, 87.5% of the isolates were resistant to Cotrimoxazole and Ciprofloxacin and 78.6% of the isolates were resistant to Amikacin. With the exception of Imipenem, more than 70% of the isolates exhibited resistance to the remaining antibiotics tested (Fig. 2).

Plasmid Profile Analysis

Plasmid profile analysis of all the *Pseudomonas aeruginosa* isolates was analyzed in 0.8% gel to understand the possible link with the antibiotic resistance properties. In this study, all the isolates showed the presence of fragmented chromosomal DNA of identical size. The agarose gels for resolution showed no plasmid DNA from the isolates (Fig. 3).

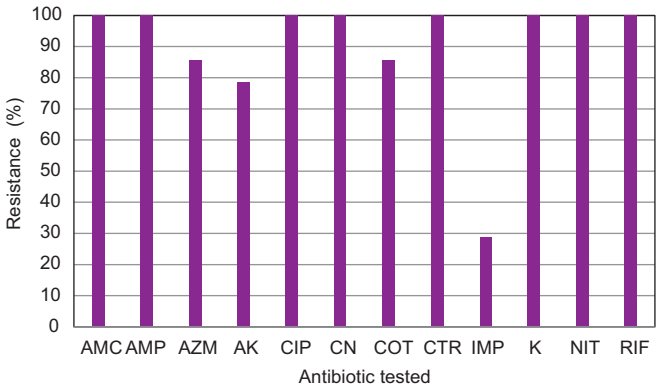

Fig. 2. *Antibiotic resistance pattern of the isolates to different antibiotics. Keys to names of antibiotics are: AMP – Ampicillin, COT - Co-Trimoxazole, AMC - Amoxycillin-Clavulanate, IPM – Imipenem, RIF – Rifampicin, AZM - Azithromycin, CN – Cephalexin, CIP – Ciprofloxacin, CTR – Ceftriaxone, S – Sensitive, AK – Amikacin, I – Intermediate, K – Kanamycin, R - Resistant*

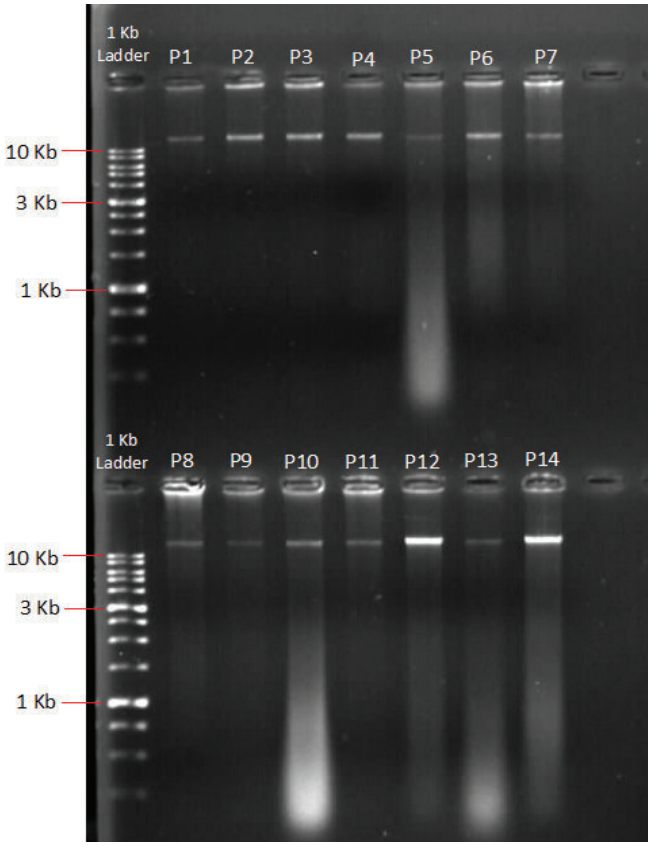

Fig 3. *Agarose gel electrophoresis of isolates showing respective band patterns following plasmid extraction. Only chromosomal fragment was observed. The ladder is 1 kb ladder from GeneON (Germany)*

Presence of Class 1 Integron gene in the *Pseudomonas aeruginosa* isolates

In this study, 50% of the isolates (n=14) contained Class 1 Integron (Fig 4). All Class 1 Integron bearing isolates showed similar antibiotic resistance profiles, indicating to the possibility of having

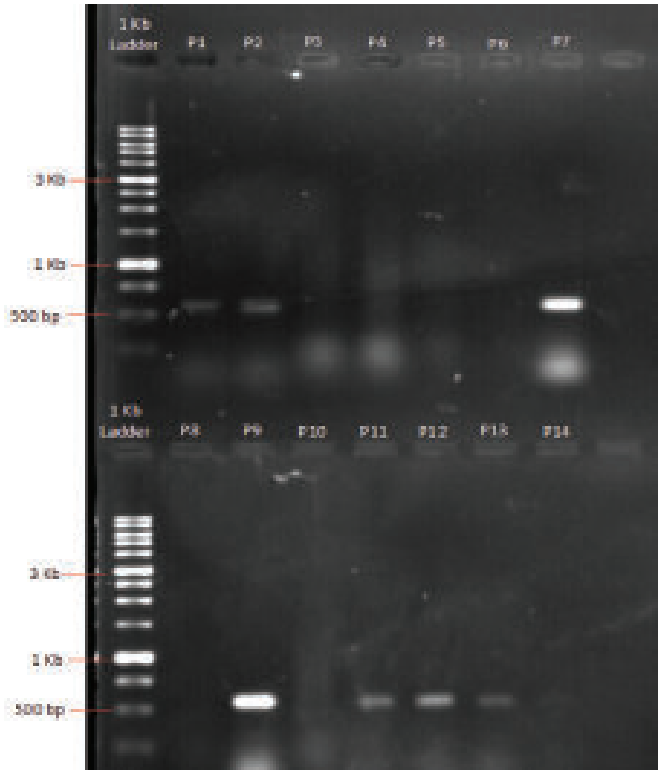

Fig. 4. *Presence of Class 1 integron in Pseudomonas aeruginosa. Lane 1 is 1Kb ladder from Gene ON. Lanes P1-P14 denote isolates 1-14.*

similar gene cassettes in Integrons. Isolates that contained Class 1 Integron showed variation in resistance to Imipenem only.

Discussion

Antibiotic resistance is a burning issue in the field of medicine[25]. One of the mechanisms by which Gram-negative bacteria acquire antibiotics is the transfer of antibiotic resistance genes via mobile genetic elements. The present study was undertaken to investigate the antibiotic resistance pattern and the presence of mobile genetic elements, particularly plasmids and Class 1 Integron gene, in 14 clinical isolates of *P. aeruginosa*.

In this study, the isolates showed resistance to most of the antibiotics tested, with the exception of Imipenem. Although plasmid was not observed in none of the isolates, 50% of the isolates carried Class 1 Integron. The presence of mobile genetic element is an added threat of antibiotic resistance as this may enable transfer of resistance genes to sensitive isolates.

Overuse and misuse of antibiotics, disposal of clinical waste containing antibiotics in the environment, lack of proper education and large scale use in aquatic culture and livestock are increasing the selective pressure that favor the antibiotic resistant organisms throughout the world including Bangladesh[26-29]. The fact that all the isolates were MDR in this study emphasized the need towards controlled use of antibiotics for the treatment of *Pseudomonas* infections. It is high time to study the prevalence of MDR *P. aeruginosa* in Bangladesh. Our results were, however,

different from that of Safain *et al.* (2018)[30] who found lower rate of resistance among *P. aeruginosa* isolates (n=17) from different environments. In an earlier study, Ameen *et al*. (2015)[31] found complete susceptibility of test isolates (n=32) from surface water to the 3rd generation antibiotics (Beta-lactams, Ciprofloxacin, etc.) which is in contrast to our findings. In case of street food sold in Dhaka city, majority of the *Pseudomonas* isolates were found to be resistant to Nitrofurantoin[32] , a finding different from our study where all of the isolates were resistant to this drug. In a study conducted on clinical isolates in 2007 (n=294) from Dhaka Medical College Hospital, Imipenem was found to be a drug of choice for the treatment of *P. aeruginosa*. In our study, only 28.5% (n=4) of our isolates were susceptible to Imipenem, hence in complete contrast with the aforementioned study. Although the number of sample studied in our case was much smaller, the increasing trend of resistance to different antibiotics is alarming and again calls for alternative treatment strategies.

Plasmids play critical roles in bacterial fitness and evolution of *P. aeruginosa*. In spite of this, none of the 14 isolates contained plasmid. In *P. aeruginosa* clinical isolate HS87, two plasmids were found and their sizes were 11.2kb and 26.8 kb[33]. These plasmids can carry mobile genetic elements and antibiotic resistance genes resulting in the evolution of MDR *P. aeruginosa.* However, the same authors also reported that no plasmid has been seen in the complete genome of *P. aeruginosa*, a statement in concordance with our findings.

Antibiotic resistance genes in *P. aeruginosa* are often associated with mobile genetic elements such as transposons, integrons and IS elements[34-39]. In our study, we found Class 1 Integron gene in 50% of the isolates. However, there was no specific correlation between occurrence of multi-drug resistance and presence of integron gene, indicating that the chromosome played a crucial role in the antibiotic resistance.

Conclusion

The detection of antibiotic resistance profile of a pathogen is important, particularly in the context of treatment. The present study indicates the presence of multiple antibiotic resistant *P. aeruginosa* in clinical samples. The major limitation of this study, however, was the small number of bacterial isolates and the lack of their molecular identification. The spread of these bacteria can lead to endemic outbreaks and the lack of awareness to treat these bacteria appropriately may confer resistance of these bacteria to more types of antibiotics. However, conjugation experiments and chemical transformation were not conducted in the present investigation to determine plasmid transfer. Whether gene transfer occurs naturally remains the focus of future studies. Hence, care has to be taken against treating diseases involving them. The study also identifies that all the bacteria are still sensitive to Imipenem. However, a fair assumption is that, if misuse and overuse of antibiotics in Bangladesh is not reduced then the effective antibiotics would not remain effective for long.

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