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Alarming Trend of Antibiotic Resistance in *Pseudomonas aeruginosa* Isolates

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Pseudomonas aeruginosa **is a concerning opportunistic pathogen frequently causing nosocomial and life-threatening infections. The present study was thus conducted to determine the prevalence of antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa* in Egypt. From 250 clinical specimens, 86 isolates of *P. aeruginosa* (34.4%) were isolated to assess the level of antimicrobial susceptibility and to determine the possible existing resistance mechanisms to commonly used antibiotics. It was found that piperacillin, meropenem, amikacin and polymyxin B were the most effective antibiotics against *P. aeruginosa* followed by imipenem, ticarcillin, ciprofloxacin, ceftazidime, cefipime, gentamicin and norfloxacin. *P. aeruginosa* isolates were highly resistant to all other antibiotics tested. Mechanisms of resistance used by *P. aeruginosa* included β-lactamase production and multiple drug resistance efflux pumps. The present results showed that 42 (48.8%) of the clinical *P. aeruginosa* isolates were β-lactamase producers. In addition, efflux pump was identified in 34 (39.5%) of *P. aeruginosa* isolates that effectively utilized an efflux-mediated mechanism of resistance against cefotaxime, ticarcillin, azetreonam, meropenem and norfloxacin but not to streptomycin. In conclusion, antibiotic resistance in clinical isolates of *P. aeruginosa* could be attributed to β-lactamase production and the use of multiple drug resistance efflux pumps.**

Key words: *Pseudomonas aeruginosa*, Multidrug resistance, β-lactamases, Efflux pumps.

Pseudomonas aeruginosa is a versatile Gram negative opportunistic human pathogen, which is becoming increasingly more prevalent. It is associated with infections of immunocompromised individuals, as a result of burns or other severe trauma, underlying diseases, including cancer, diabetes, cystic fibrosis, and deliberate immunosuppression[32]. The pathogenesis of *P. aeruginosa* is multifactorial as underlined by the large number of virulence factors and the broad spectrum of diseases the bacterium causes[8]. *P. aeruginosa* causes both community acquired infections as well as severe nosocomial infections, life threatening infections in immunocompromised persons, and chronic infections in cystic fibrosis patients[26, 27]. A major reason for its prominence is its high intrinsic resistance to antimicrobial agents, such that even for the most recent chemotherapeutic antimicrobial agents, a modest change in susceptibility can prevent their effectiveness[39]. The prevalence of *P. aeruginosa* in hospitals owes much to the intrinsic resistance of the organism to multiple antimicrobial agents[21]. Many problems caused by *P. aeruginosa* are partly because the organism is inherently resistant to many drug classes and are able to acquire resistance to most effective antimicrobial drugs.[13, 38]

Therefore, it is important to study the resistance patterns of *P. aeruginosa* isolates to commonly used antibiotics. The use of antibiogram as an epidemiological indicator can help us make the best use of antibiotics in the treatment of *P. aeruginosa* infections. The prevalence of *P. aeruginosa* infection in hospitalized patients was studied. The increasing antibiotic resistance of this

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organism is attributed to multiple factors including active drug efflux and β-lactamase production. [16] Thus, the study was conducted by testing these two possible mechanisms of resistance in *P. aeruginosa* isolates.

MATERIAL AND METHODS

Bacterial isolates

Two hundred and fifty clinical specimens were collected from inpatients and outpatients admitted to the Zagazig University Hospitals and Zagazig Chest Hospital over a fifteen-month period. All samples were collected from patients with clinically diagnosed urinary tract infections (UTI), respiratory tract infections (RTI), infected wounds and ear infections. Specimens were taken as urine sample from patients with UTI, pus swabs or sputum were collected according to type of infection. The specimens were screened for the presence of *P. aeruginosa* as a causative agent according to standard microbiological and biochemical procedures.

Antibiotics

The following antibiotics were used: ampicillin, amoxicillin, cefotaxime, tetracycline, norfolxacin, ceftriaxone , and erythromycin (from Egyptian International Pharmaceutical Industries, Cairo, Egypt), amoxicillin/clavulanate (Medical Union Pharmaceutical, Ismailia, Egypt), streptomycin and doxycycline (Nile Pharmaceutical, Cairo, Egypt), kanamycin (Misr Pharmaceutical, Cairo, Egypt), cefuroxime and ceftazidimie (Glaxo Wellcome, Cairo, Egypt), aztreonam, cefepime, amikacin (Bristol Myers Squibb, Cairo, Egypt), gentamicin (Memphis Pharmaceutical and Chemical, Cairo, Egypt), cefoperazone, azithromycin (Pfizer, Egypt), imipenem (Merck Sharp and Dohme, Hertfordshire, U.K.), meropenem (Astra-Zeneca, Cheshire, U.K.), and chloramphenicol (Chemical Industries Development, Cairo, Egypt). Cloxacillin, ticarcillin, piperacillin, and potassium clavulanate (Sigma-Aldrich, St Louis, MO, USA), polymyxin B (Novo Industry A/S, Copenhagen, Denmark), and ciprofolxacin (Bayer AG, Wuppertal, Germany).

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) (μg/ml) of different antibiotics were determined by standard serial twofold dilution method using Mueller-Hinton agar (MHA),[4] according to Clinical Laboratory Standards Institute guidelines (CLSI)[42]. Briefly, overnight cultures of bacteria in Mueller-Hinton broth (MHB) were adjusted to 10^7 cfu/ml with fresh MHB. Approximately 10^5 cfu were spotted onto the dried surface of MHA plates containing gradient dilutions of the selected antibiotics (0.125 to 1024 μg/ml). Plates were incubated at 37°C for 18-20 h and MICs were determined. MIC was defined as the lowest concentration of antibiotic showing no visible growth.

Detection of β-lactamase

Nitrocefin is a chromogenic cephalosporin used to detect β-lactamases as described previously[24]. A 0.5 mM solution was prepared by dissolving nitrocefin powder (Glaxo, Middlesex, U.K.) in 0.1 M phosphate buffer saline (PBS), pH 7 containing dimethylsulfoxide (DMSO). Colonies of the test isolates were scraped from nutrient agar plates and suspended into 20 μL of PBS to produce a dense suspension on a glass slide followed by the addition of μL of nitrocefin solution. β-lactamase activity was indicated by the development of red color within 1-2 min.

Study of efflux systems

The existence of efflux mechanism in *P. aeruginosa* isolates was determined by testing the accumulation of ethidium bromide in the presence or absence of efflux inhibitors.[29] Overnight cultures were adjusted to approximately 10^5 cfu. Washed harvested cells were resuspended in 20 μL of 1 μg/ml ethidium bromide with or without either 100 μM dinitrophenol (DNP, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) or 0.4 % glucose or 0.1 % of toluene and were incubated at 37°C for 15 min. Cells were collected by centrifugation at 1200 *x g* for 5 minutes and re-suspended in 10 μl of PBS. Cell suspensions (in 5 μL amounts) were spotted onto the surface of 1% agarose gel and examined over UV-transilluminator. Drug accumulation in *P. aeruginosa* cells was observed as bright fluorescence of ethidium bromide.

To study the efflux system of *P. aeruginosa* isolates, the MICs of antimicrobial agents for 37 MDR *P. aeruginosa* isolates were determined in the presence and absence of 100 μM of the efflux pump inhibitor DNP and dicyclohexylcarbodiimide (DCCD, Sigma-Aldrich,

Steinheim, Germany). [4] The reduction in MIC of a certain antibiotic with DNP or DCCD is an indication of resistance to this antibiotic mediated by an efflux system.

RESULTS

Prevalence of *P. aeruginosa* in clinical specimens

Out of the 250 clinical specimens, 86 *P. aeruginosa* isolates (34.4%) were isolated and identified biochemically using standard procedures for detection.

As shown in Table 1, 20 (23.8%) isolates were from 84 specimens of sputum (33.6%), 23 (51.1%) were from 45 wound exudates (18%), 30 (53.6%) from 56 urine in case of UTI (22.4%), and 13 (20%) were from ear discharges (26%).

Antibiotic susceptibility and determination of MICs

Table 2 shows the respective MIC distributions of different antibiotics for 86 clinical isolates of *P. aeruginosa*. Table 3 shows the MIC_{90} (MIC required to inhibit the growth of 90% of organisms) of each antibiotic, and whether the bacteria were susceptible, intermediately susceptible or resistant to each antibiotic. The breakpoint MIC of an antibiotic is the highest concentration that can be safely administered to the patients. Organisms are considered susceptible to an antibiotic if the MIC is below the breakpoint MIC and considered resistant to antibiotic if the MIC exceeds the breakpoint MIC of that antibiotic.

Among the antibiotics tested, piperacillin, meropenem, amikacin and polymyxin B were the most effective antibiotics against clinical isolates of *P. aeruginosa* followed by imipenem, ticarcillin, ciprofloxacin, ceftazidime, cefipime, gentamicin and norfloxacin. *P. aeruginosa* isolated strains were highly resistant to all other antibiotics tested. As shown in Table 3, all of 86 clinical isolates of *P. aeruginosa* were resistant to more than three antibiotics and were defined as MDR.[25, 30]

Resistance through β-lactamase production

All *P. aeruginosa* isolates were subjected to β-lactamase detection. A color change of nirocefin from yellow to red indicated β-lactamase production as described in materials and methods section. The present data showed that 42 (48.8%) of the clinical *P. aeruginos*a (86 isolates) were β-lactamase producers.

Resistance through the efflux system

The existence of efflux activity in *P. aeruginosa* isolates was studied by the accumulation of ethidium bromide. Reduction in fluorescence intensity was observed with MDR isolates in the absence of efflux pump inhibitor and in presence of glucose (acts as an energizer of efflux pump). In the presence of efflux pump inhibitor or toluene (as a membrane permeabilizer) there was an increase in fluorescence intensity. *P. aeruginosa* ATCC 90271 was used as negative control. It was found that 34 isolates (39.5%) of clinical *P. aeruginosa* (86 total isolates) were positive for efflux pump existence.

Table 4 shows the MICs of six antibiotics (cefotaxime, ticarcillin, azetreonam, meropenem, norfloxacin and streptomycin) for 37 different MDR *P. aeruginosa* isolates in the presence and absence of the efflux inhibitors (DNP, and DCCD). The addition of DNP and DCCD enhanced the activities of selected antibiotics as observed in the reduction of MIC. These results emphasized the existence of an efflux-mediated resistance in the tested isolates to cefotaxime, ticarcillin, azetreonam, meropenem and norfloxacin but not to streptomycin.

DISCUSSION

P. aeruginosa is an emerging pathogen of concern due to its increasingly reported resistance. *P. aeruginosa* isolates show steady escalation in resistance to an increasing number of antimicrobial agents and the emergence of MDR *P. aeruginosa* infections is increasingly recognized. The present study observed that *P. aeruginosa* represented 34.4% of all clinical specimens collected (Table 1), and all of them have absolute susceptibility to polymyxin B, amikacin, meropenem, and pipracillin (Table 3) as has been reported previously[11, 33, 43].

The resistance rate to imipenem was 1% similar to other studies [3, 18] while different resistance rates were reported in different studies showing higher resistance rates ranging from 9.5% from Japan [31] to 42.3% from Poland [37]. All tested isolates were sensitive to meropenem, although higher resistance rate (45.5%) was reported in studies from Bulgaria[37] and (46%) USA[36]. Similarly, all *P. aeruginosa* isolates in the present study were sensitive to piperacillin while other reports showed

high resistance rates[10, 23]. Other studies showed an emergence of resistance of *P.aeruginosa* isolates to polymyxin B[17, 22]. Also resistance rates to amikacin were reported in previous studies ranging from 4.2 % in Saudi Arabia [2] to 59.1% in Bulgaria[37], 64% in USA[36], and 77.1% in Iran[19].

The present data revealed resistance rates against aztreaonam, ticarcillin, and gentamicin similar to other studies[43]. In the present study, ceftazidime was the most active cephalosporins with a susceptibility rate of 58% and this is consistent with other reports[12, 34]. This was followed by cefepime with a susceptibility rate of 40% as similar to previous studies[23, 36] while ceftraixone showed resistance rate of 29% which was lower than that reported by other studies[6, 23]. Cefotaxime showed the highest resistance rate of 58% similar to other reports with higher resistance rates.[15, 28] All isolates were resistant to cefoperazone and cefuroxime. The resistance pattern to cephalosporins was consistent with Yetkin *et al.,*[43] who reported that the resistance rate to cephalosporins was in the range of 27% to 88%.

The present results revealed that resistance rate of *P. aeruginosa* to ciprofloxacin were 34%, while a previous study [7] reported that *P. aeruginosa* was absolutely susceptible to ciprofloxacin. In yet another study the reported resistance rate to ciprofloxacin was 11.9% in 1999 and 20.6% in 2006[34]. In the present study, resistance rate to norfloxacin reached 86.5% similar to other studies[6, 35]. This discrepancy of resistance rates can be attributed to the continuous development of MDR strains of *P. aeruginosa* in different parts of the world.

In order to determine the possible mechanisms by which *P. aeruginosa* isolates resist antibiotics, the isolates were tested for β-lactamase production and efflux-mediated resistance. *P. aeruginosa* were previously shown to use β-lactamase-mediated resistance to antibiotics[5,40,41]. It was observed that high levels of β-lactamase production in *P. aeruginosa* clinical isolates (48.8%)which was similar to the data previously reported[11, 14] . It was observed that the reduction of MICs of cefotaxime, ticarcillin, aztreaonam, meropenem in the presence of efflux pump inhibitors (DNP and DCCD) indicating the presence of efflux-mediated resistance by *P. aeruginosa* isolates. This is similar to other reports which showed major contribution of efflux in the emergence of resistance in *P. aeruginosa*[1,9,11,20,38,44]. Therefore, the possible existing mechanisms of resistance of *P. aeruginosa* isolates include β-lactamase production and the use of multiple drug resistance efflux pumps.

CONCLUSION

Although data presented in this study showed that resistance of clinical isolates of *P. aeruginosa* to commonly used antibiotics is high, the importance of the results is indicating that escalating rates of MDR among isolates still pose a clinical problem for patients and health officials. These high resistance rates could be attributed to extensive usage of antibiotics and the contribution of different mechanisms to the current resistance levels of *P. aeruginosa*. It is suggested that, to minimize the impact of resistance and its spread, a implementation of a regional and nationwide surveillance program be instituted to monitor antimicrobial resistance trends among *P. aeruginosa*. For practicing physicians, clinical microbiologists, and public health officials, knowledge of antibiotic resistance patterns is essential to guide empirical therapy. In addition, preventive strategies such as continuous surveillance of *P. aeruginosa* resistance against antimicrobial agents, prudent antimicrobial use and infection control should be advocated to delay emergence of clinically significant MDR *P. aeruginosa*. Also, emphasis should be made on the importance of legislation of usage of antibiotics. This requires an urgent need for control policy of prescription and use of antibiotics in hospitals for the prevention of the steady increase in *P. aeruginosa* resistance. In addition, this alarming trend of resistance deserve attention and concern among health care providers and requires continuation of surveillance studies worldwide to control antibiotic resistance. Futhermore, search for new antimicrobial agents to bypass the steady resistance of *P. aeruginosa* to currently used antibiotics.

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