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Comparative study of virulence factors among ESβL-producing and nonproducing *Pseudomonas aeruginosa* clinical isolates

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Background/aim: β-Lactamase production is considered one of the most important resistance mechanisms among virulent *Pseudomonas aeruginosa* isolates. The aim of this study was to compare the production and antimicrobial resistance patterns of some virulence factors in extended spectrum β-lactamase (ESβL)-producing and nonproducing *P. aeruginosa* clinical isolates.

Materials and methods: Out of 183 different clinical specimens, 104 *Pseudomonas aeruginosa* isolates were recovered. The isolates were screened for ESβL production using the double disk diffusion test and phenotypic confirmatory disk diffusion test. All isolates were tested for susceptibility to 25 antimicrobials, as well as for expression of various virulence factors including pigment, hemolysin, gelatinase, protease, lipase, rhamnolipids, biofilm, and cell surface hydrophobicity. The results of ESβL producers and nonproducers were statistically compared.

Results: All isolates showed a high frequency of multiple resistance to at least 14 and up to 25 of the tested antimicrobials. Nevertheless, most virulence factors were produced at higher rates in ESβL-producing than in ESβL-nonproducing *Pseudomonas aeruginosa* isolates.

Conclusion: The results of this study suggest a correlation between ESβL phenotype and the production of some factors that are reported to be involved in the virulence of *P. aeruginosa*.

Key words: Extended spectrum β-lactamase, *Pseudomonas aeruginosa*, double disk diffusion test, phenotypic confirmatory disk diffusion test, virulence factor

1. Introduction

Recently, multidrug-resistant gram-negative bacteria have become more prevalent and are causing great problems in the treatment of infections (1). *Pseudomonas aeruginosa* is a gram-negative rod bacterium, which is reported to be ubiquitous in humans, animals, and the natural environment. The widespread habitat of *P. aeruginosa* makes it very difficult to control the organism in a hospital setting. Prevention of contamination is practically impossible. The main danger is the infection of patients who are immunologically compromised or those in burn units, neonatal units, and cancer wards. *P. aeruginosa* is difficult to eradicate due to a number of factors, the most important of which is the relatively poor efficacy of antibiotics against *P. aeruginosa* due to multiple resistance mechanisms expressed by the bacterium (2). This pandrug resistance, together with high attributable mortality, has thrust *P. aeruginosa* into the spotlight as an emerging superbug (3).

One of the clinical significances of *P. aeruginosa* is its ability to secrete several virulence factors. These virulence factors include mucoid exopolysaccharide, lipopolysaccharide, biofilm, pili, exotoxin A, pigments, lipase, protease, hemolysin, histamine, exoenzyme S, leukocidin, and rhamnolipids (4). These factors help the bacteria to adhere to and invade their host by damaging the host's immune responses and forming a barrier to antibiotics. Cell-associated and secreted virulence factors are encoded on plasmids or chromosomal genes (2).

From another point of view, *P. aeruginosa* is notorious for its resistance to antibiotics and therefore is a particularly dangerous and dreaded pathogen. It is one of the leading causes of severe infections, such as pneumonia or bacteremia, which are associated with high mortality rates and are often difficult to treat. Reports of useful antipseudomonal agents are limited (some β-lactams, fluoroquinolones, aminoglycosides,

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and polymyxins as last-resort drugs) and *P. aeruginosa* exhibits high intrinsic resistance to penem antibiotics such as faropenem, ritipenem, sulopenem, tetracycline, and penicillins (5,6). The persistent exposure of bacterial strains to a multitude of β-lactams has induced a dynamic and continuous production and mutation of β-lactamases in the bacteria, expanding their activity even against the third and fourth generation cephalosporins, such as ceftazidime, cefotaxime, and cefepime, and also against aztreonam. These new β-lactamases are called extended spectrum β-lactamases (ESβLs) (7). The methods for detection of ESβLs can be broadly divided into 2 groups: phenotypic methods that use nonmolecular techniques, which detect the ability of the ESβL enzymes to hydrolyze different cephalosporins; and genotypic methods, which use molecular techniques to detect the gene responsible for the production of the ESβLs (8). Clinical diagnostic laboratories use mostly phenotypic methods (double disk diffusion test [DDDT], phenotypic confirmatory double disk test [PCDDT], and ESβL E-test) because these tests are easy to perform and are also cost effective. The present study aims to detect any possible correlation between the production of virulence factors and antibiotic resistance among *P. aeruginosa* clinical isolates. We also tested whether ESβL-producing *P. aeruginosa* possess distinguishing virulence characteristics when compared to their non-ESβL-producing counterparts, which might have implications for treatment and control.

2. Materials and methods

2.1. Collection of specimens

A total of 183 clinical specimens were randomly collected in sterile screw cupped containers from patients attending the clinics of the Tanta University hospitals, Egypt, from May 2010 to April 2011. These included 35 burn swabs, 18 ear swabs, 11 catheter tip swabs, 48 urine samples, 9 blood samples, and 62 samples from sputum. Specimens were immediately placed in nutrient broth transport media and then transferred to the bacteriology laboratory of the Department of Botany, Faculty of Science, Tanta University, Egypt.

2.2. Isolation and identification of bacterial isolates

Each specimen was cultured on blood agar and nutrient agar plates. The resultant colonies in these media were subcultured on MacConkey agar. Nonlactose fermenting colonies were further subcultured on cetrimide agar plates supplemented with 15 μg/mL nalidixic acid for preliminary selection of *Pseudomonas aeruginosa* isolates. The recovered isolates were subjected to different morphological and biochemical tests for identification to the species level as described by *Bergey's Manual for Systematic Bacteriology* (9). The isolates were identified as *Pseudomonas aeruginosa* on the basis of typical morphology by gram-negative staining, growth at 42 °C but not at 4 °C, and positive oxidase, arginine, and catalase reactions. The isolates were all oxidative organisms when grown on Hugh and Liefson medium and were able to reduce nitrate to nitrite. Identification of tested isolates was confirmed with an API 20E (bioMérieux) identification kit. Stock cultures were stored in 0.05 M K-Na-phosphate buffer, pH 7.0, containing 15% glycerol at –20 °C.

2.3. Antimicrobial susceptibility testing

All tested *P. aeruginosa* isolates were screened for their susceptibility to 25 different antimicrobials, representing 11 classes, using the disk agar diffusion method (a modified Kirby–Bauer method) on Mueller-Hinton agar media following the zone diameter criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) (10). For the purpose of analysis, isolates with resistant phenotypes included those that were classified as intermediate resistant.

2.4. Detection of some virulence factors

2.4.1. Pigment production

The tested isolates were streaked on Fluka *Pseudomonas* isolation agar F (PIA F) to detect pyoverdin (fluorescein) production and Fluka *Pseudomonas* isolation agar P (PIA P) to detect pyocyanin production. Observation of any colors produced by the isolates was recorded (11).

2.4.2. Hemolysin production

Tested isolates were cultured on sheep blood agar media as described by Pavlov (12). Plates were incubated at 37 °C for 24 h and then checked for a zone of hemolysis around colonies. The results were recorded as follows: α-hemolysis (greenish zones), β-hemolysis (clear zone), or γ-hemolysis (no hemolysis).

2.4.3. Gelatinase activity

Gelatin production character was tested in bacterial inoculation tubes containing nutrient gelatin medium (13). The tubes were incubated for 48 h at 37 °C. Uninoculated tubes were run besides the inoculated ones as negative controls. At the end of the incubation period, the liquefaction of the culture medium was observed after placing the culture tube at 4 °C overnight.

2.4.4. Protease activity

All the tested *P. aeruginosa* isolates were assayed for protease production ability using skimmed milk agar as described by Madigan (14). Tested isolates were streaked on the 2% skimmed agar and incubated at 37 °C for 24 h. Incubated plates were checked for halo regions around streaks, and a zone of clearing of more than 1 mm around the streaks was recorded as positive for protease production.

2.4.5. Lipase activity

Tween 80 agar plates were inoculated with each tested isolate and incubated at 37 °C for 1–7 days. After

incubation, opaque zones surrounding the inocula were indicative of Tween 80 hydrolysis (12).

2.4.6. Rhamnolipid production

The presence or absence of rhamnolipids was assessed by the rhamnolipid-mediated alkane degradation method as described by Zulianello (15) using a minimal supplement agar plate for cetyltrimethylammonium bromide (CTAB) assay. Two microliters of each *P. aeruginosa* overnight culture was spotted on the detecting plates and allowed to dry prior to incubation at 37 °C for 24 h. Plates were then incubated at 25 °C for a further 48 h. *P. aeruginosa* that produced rhamnolipids appeared with a blue halo surrounding the colony.

2.4.7. Biofilm formation

Biofilm production by *P. aeruginosa* was estimated qualitatively for all the isolates by the tube method as described previously by Christensen et al. (16). After incubations at 37 °C for 18–20 h, the cultures were decanted. Tubes were stained with safranin and the presence of a visible stained film lining the wall and bottom of the tube was considered positive for slime production. Ring formation at the liquid interface was not indicative of biofilm formation.

2.4.8. Cell surface hydrophobicity

Microbial surface hydrophobicity was assessed with xylene according to Rosenberg and Gutnick (17). All isolates were grown in a nutrient broth (50 mL) in a 250-mL Erlenmeyer flask shaken at 200 rpm. Cells were harvested by centrifugation (10,000 rpm, 15 min), washed twice in sterile phosphate-buffered saline (pH 7.1), and suspended in the same buffer to an initial optical density (OD) of about 1.0 (A_0) at 600 nm. An aliquot of 300 µL of xylene was added to 3 mL of bacterial suspension and vortexed for 2 min. After standing for 10 min, the OD of the aqueous phase was measured (A_1) at 600 nm. The degree of hydrophobicity was calculated as $[1 - (A_1/A_0)] \times 100$ (%); cell surface hydrophobicity (CSH) was considered weak at 0%–20%, moderate at 21%–50%, and strong at >50%, as explained by Rosenberg and Gutnick (17).

2.5. ESβL detection

All isolates were tested for their susceptibility to third generation cephalosporins (3GCs) ceftazidime (30 µg/disk), cefotaxime (30 µg/disk), and ceftriaxone (30 µg/disk) by using the standard disk diffusion method as recommended by the CLSI (10). Isolates that were resistant to at least 1 of the 3GCs were selected for study and were processed for ESβL production. If a zone diameter of ≤22 mm for ceftazidime, ≤27 mm for cefotaxime, or ≤25 mm for ceftriaxone was recorded, the isolate was considered to be suspicious for ESβL production (10). All isolates were confirmed for ESβL production using 2 phenotypic methods, as described below.

2.5.1. DDDT method

An augmentin (20 µg of amoxicillin and 10 µg of amoxicillin/clavulanic acid [AMC]) disk was placed in the center of a Mueller-Hinton agar plate and inoculated with the tested organism, and disks of the above 3GCs were placed 20 mm away from the AMC disk. The plate was incubated overnight at 37 °C. ESβL production was considered positive if the inhibition zone around 1 or more cephalosporin disks was extended on the side towards the AMC disk, appearing as a ghost zone (18).

2.5.2. PCDDT method

Ceftazidime and cefotaxime disks (30 µg), alone and in combination with clavulanic acid (30/10 µg), were applied onto plates of Mueller-Hinton agar and inoculated with the tested isolates. The diameter of the zone of inhibition was measured after overnight incubation at 37 °C. An increase of ≥5 mm in diameter in a zone of inhibition of the combination disks in comparison to the cefotaxime or ceftazidime was considered to be a marker for ESβL-producing isolates (10).

2.6. Statistical analysis

Statistical presentation and analysis of the present study was conducted, using the mean, standard error, chi-square, linear correlation coefficient, and analysis of variance [ANOVA] tests by SPSS, version17.

3. Results

In the present study a total of 104 (56.8%) *P. aeruginosa* isolates were recovered from clinical specimens. Isolates were distributed among samples as shown in Table 1.

3.1. Susceptibility of *P. aeruginosa* isolates to different commonly used antimicrobial agents

The obtained data showing the incidence of antimicrobial resistance among recovered isolates are presented in Figure 1. Generally, the incidence of resistance to different tested antibiotics ranged between 18.3% (tobramycin) and 100% (ampicillin, amoxicillin, amoxicillin/clavulanic acid, cefepime, ceftriaxone, ceftizoxime, kanamycin, and chloramphenicol).

The resistance patterns of the tested isolates were noticeably heterogeneous; a total of 13 patterns were detected among the 104 tested isolates (data not shown). The most common resistance pattern was pattern S (ampicillin, amoxicillin, piperacillin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, ceftazidime, cefepime, cefotaxime, ceftriaxone, ceftizoxime, cefoperazone, imipenem, meropenem, aztreonam, gentamicin, amikacin, streptomycin, tobramycin, kanamycin, ciprofloxacin, cotrimoxazole, tetracycline, chloramphenicol, and colistin sulfate), which was exhibited by 14 (13.5%) isolates. All isolates showed a high frequency of multiple drug resistance (n = 14–25), and up

Table 1. Distribution of *P. aeruginosa* isolates in different clinical sources.

Specimen/swabs	Number of specimens	Number of recovered isolates	Percent of total isolates
Sputum	62	43	41.3%
Urine	48	30	28.8%
Burn	35	20	19.2%
Ear swab	18	4	3.8%
Blood	9	4	3.8%
Catheter tip	11	3	2.9%
Total	183	104	100%
Chi-square	χ^2	80.1	
	P-value	<0.001*	

*: P ≤ 0.05 considered significant.

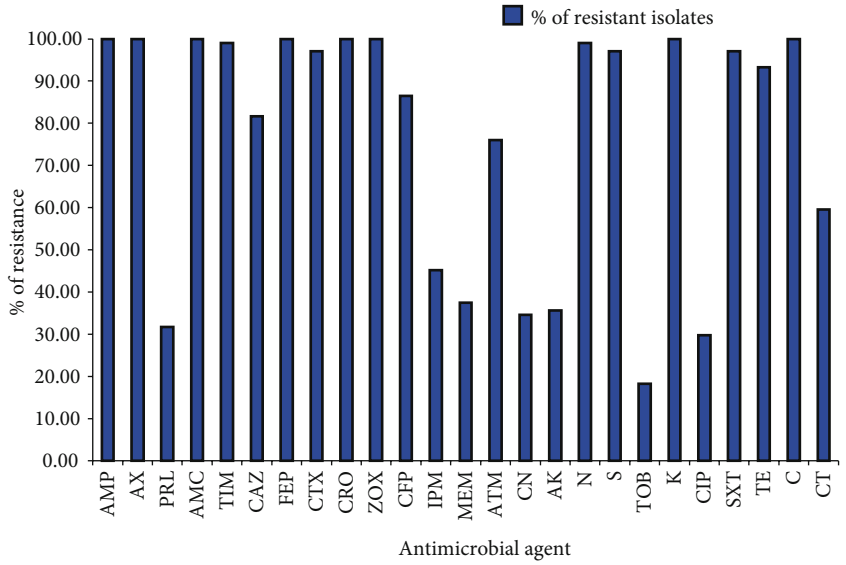

Figure 1. Histogram showing susceptibility of *P. aeruginosa* to tested antimicrobial agents. AMP, ampicillin; AX, amoxicillin; PRL, piperacillin; AMC, amoxicillin/clavulanic acid; TIM, ticarcillin/clavulanic acid; CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; CRO, ceftriaxone; ZOX, ceftizoxime; CFP, cefoperazone; IPM, imipenem; MEM, meropenem; ATM, aztreonam; CIP, ciprofloxacin; SXT, cotrimoxazole; TE, tetracycline; C, chloramphenicol; CN, gentamicin; AK, amikacin; N, neomycin; S, streptomycin; TOB, tobramycin; K, kanamycin; CT, colistin sulfate.

to 50% of the isolates showed multiple resistance to ≥19 antimicrobial agents.

3.2. Phenotypic expression of some virulence factors among the tested *P. aeruginosa* isolates

The obtained data showing the incidence of virulence factor production among the tested *P. aeruginosa* isolates are presented in Table 2. One hundred (96.1%) of the isolates produced pigment, ranging from 93% (sputum isolates) to 95% (burn isolates) and 100% (urine, blood, ear, and catheter tip isolates). For hemolysin production, 95.2% of the isolates showed hemolytic activity. Eighty-one (78%) of the isolates produced gelatinase. For the protease activity, the lowest percentage of protease activity was recorded in isolates from urine (73.3%), followed by sputum (83.7%).

Table 2. Distribution of virulence factors of *P. aeruginosa* in relation to clinical sources.

Sample source	No. of *P. aeruginosa* isolates	No. (%) of virulence factor producers									
		Pigment	Hemolysin	Gelatinase	Protease	Lipase	Rhamnolipids	Biofilm	Cell surface hydrophobicity (CSH)*		
									Weak	Moderate	Strong
Sputum	*43*	40 (93)	40 (93)	34 (79.1)	36 (83.7)	35 (81.4)	24 (55.8)	31 (72.1)	29 (67.4)	13 (30.23)	1 (2.32)
Urine	30	30 (100)	28 (93.3)	24 (80)	22 (73.3)	23 (76.7)	17 (63.3)	24 (80)	15 (50)	10 (33.3)	5 (16.6)
Burn	20	19 (95)	20 (100)	14 (70)	19 (95)	16 (80)	16 (80)	18 (90)	13 (65)	4 (20)	3 (15)
Ear	4	4 (100)	4 (100)	2 (50)	4 (100)	3 (75)	4 (100)	2 (50)	2 (50)	2 (50)	0 (0)
Blood	4	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	2 (50)	3 (75)	3 (75)	1 (25)	0 (0)
Catheter tip	3	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	1 (33.3)	3 (100)	0 (0)	0 (0)
Total	104	100 (96.1)	99 (95.2)	81 (78)	88 (85)	84 (81)	66 (63.5)	79 (76)	65 (62.5)	29 (27.9)	10 (9.6)
P-value		0.19	0.96	0.46	0.25	0.83	0.13	0.20	0.59		

*: CSH was determined based on the difference of the OD of bacteria before and after adsorption to hydrocarbon × 100, categorized as weak (0%–20%), moderate (21%–50%), or strong (>50%) CSH, as according to Rosenberg and Gutnick (17).

Eighty-four (81%) of the isolates produced lipase and 66 (63.5%) of the isolates were rhamnolipid producers (Table 2). With regard to biofilm formation, a total of 79 (76%) isolates were biofilm producers, and it was found that 62.5%, 27.9%, and 9.6% of isolates possessed weak, moderate, and strong CSH, respectively (Table 2).

In comparing antibiotic resistance and the detected virulence factors in *P. aeruginosa* clinical isolates, Pearson's correlation coefficient was calculated as shown in Figures 2A–2F. The obtained results show a significant correlation between antibiotics and biofilm formation ($r = 0.998$, $P < 0.0001$; Figure 2A), strong CSH formation ($r = 0.994$, $P < 0.0001$; Figure 2B), protease production ($r = 0.998$, $P < 0.0001$; Figure 2C), gelatinase production ($r = 0.997$, $P < 0.0001$; Figure 2D), lipase production ($r = 0.999$, $P < 0.0001$; Figure 2E), and rhamnolipid production ($r = 0.997$, $P < 0.0001$; Figure 2F).

3.3. Detection of ESβL producers among *P. aeruginosa* isolates and their antimicrobial resistance

ESβLs were detected by the DDDT in 15.4% of the 104 tested *P. aeruginosa* isolates. However, it was noted that the majority of tested isolates produced unsatisfactory DDDT results because they showed development of narrow zones of inhibition around the ESβL disks (Figures 3A and 3B). Isolates that showed either negative results or results that were hard to interpret by the DDDT method were further tested for production of ESβLs by the PCDDT method (Figure 3C). The ESβL producers by DDDT test were also subjected to confirmation by the PCDDT. Out of 104 tested isolates, 39 (37.5%) were ESβL producers.

Analysis of antimicrobial susceptibility revealed that resistance of ESβL producers to different β-lactams was spread markedly among the studied extended spectrum β-lactam resistant isolates and ranged between 43.6% to meropenem and 100% to ampicillin, amoxicillin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, cefepime, ceftriaxone, and ceftizoxime. Higher (100%) incidence of resistance to non-β-lactam antibiotics, namely streptomycin, kanamycin, and chloramphenicol, was also recorded in these producers as compared to the nonproducers (data not shown). Statistically, the percentages of ESβL isolates resistant to piperacillin ($P = 0.004$), ciprofloxacin ($P = 0.005$), gentamicin ($P = 0.01$), amikacin ($P = 0.01$), and tobramycin ($P = 0.01$) were significantly higher than in the non-ESβL producers. A significant correlation between ESβLs and antibiotic resistance was recorded ($r = 0.991$, $P < 0.0001$), as shown in Figure 4.

3.4. Virulence factors production among ESβL-producing *P. aeruginosa* isolates

The overall virulence factor productions among ESβL-producing (n = 39) and non-ESβL-producing (n = 65) *P. aeruginosa* isolates are shown in Figure 5. Noticeably high (100%) incidences of hemolysin (100%), pigment (100%), lipase (89.7%), biofilm (84.6%), and rhamnolipids (64.1%) were detected among the ESβL producers. All the ESβL-producing isolates were studied for presence of multiple virulence factors. A high percentage (38.5%) of ESβL producers showed production of all (n = 8) tested virulence factors studied. As the number of virulence factors increased, the rate of ESβL production in *P. aeruginosa* increased. On the other hand, all non-ESβL isolates of *P. aeruginosa* produced multiple (n = 3–8) virulence factors, ranging from 1.5% to 35.4%.

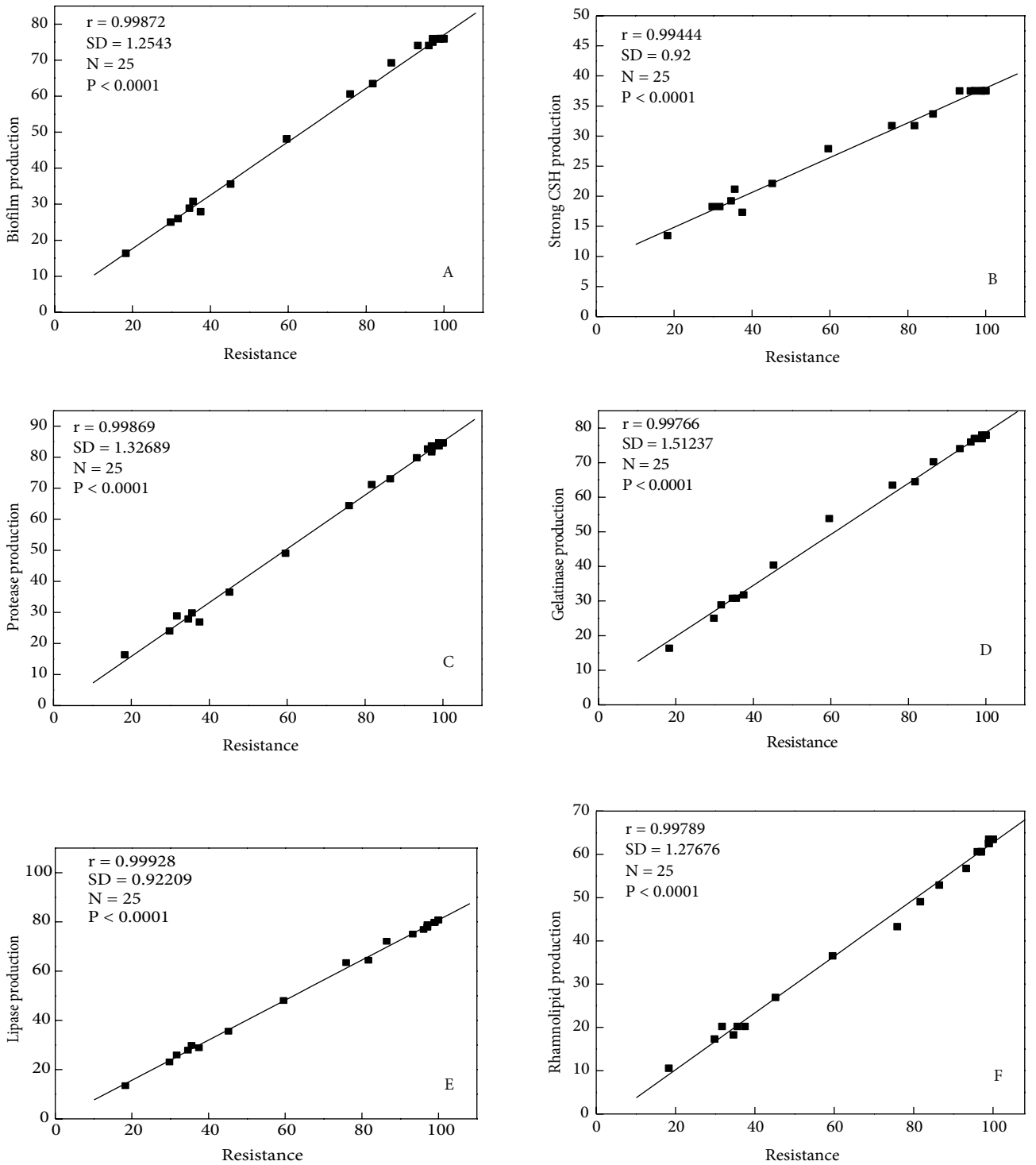

Figure 2. Correlation of antimicrobial resistance with virulence production among 104 *P. aeruginosa* isolates. N: number of antimicrobial agents; r: Pearson's correlation coefficient.

4. Discussion

With the widespread use of antibiotics and an increase in the number of immunosuppressed hosts, *P. aeruginosa* has become a leading cause of gram-negative bacterial infections, especially in immunosuppressed patients who need prolonged hospitalization (1). Moreover, *P. aeruginosa* is a serious cause of nosocomial infections. In our study, the frequency of isolates of *P. aeruginosa* varied

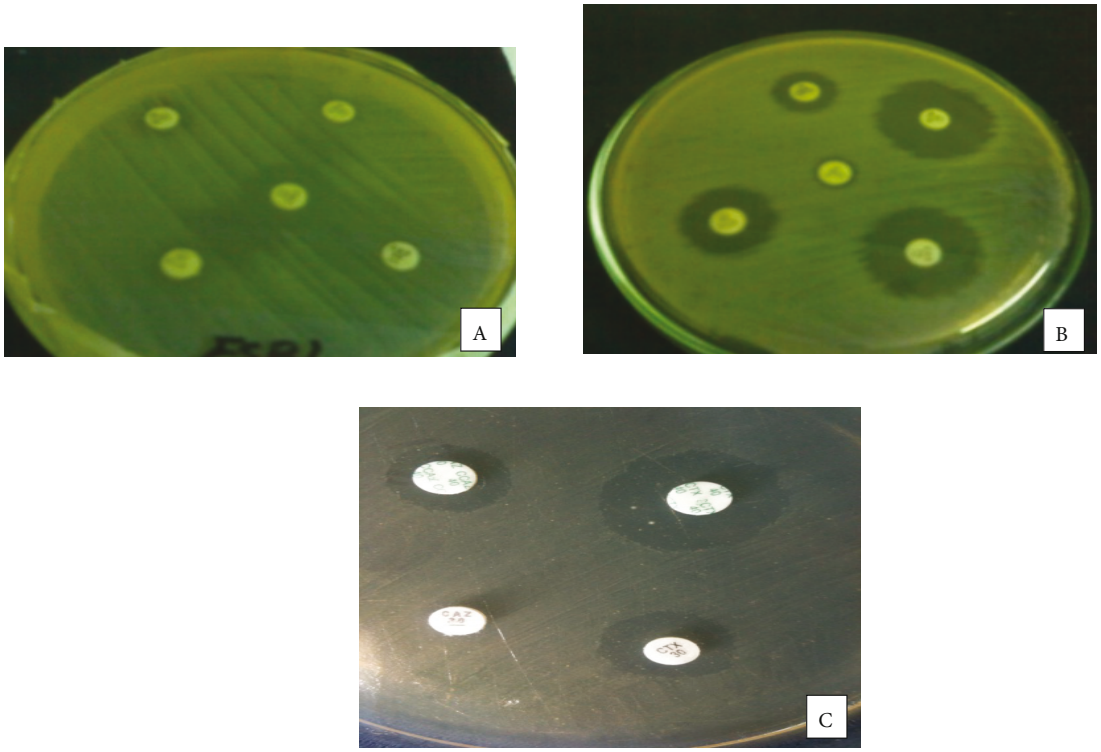

Figure 3. Detection of ESβLs by DDDT and PCDDT. **(A)** Positive result of DDDT; notice the development of a "ghost zone" between the CTX and the AMC disk in the center of the agar plat. **(B)** Negative result of DDDT. **(C)** Positive result of PCDDT; ESβL production was considered positive if there was a ≥5 mm increase in a zone diameter of an antimicrobial agent tested in combination with clavulanic acid (CCTX or CCAZ) versus its zone size when tested alone (CTX or CAZ).

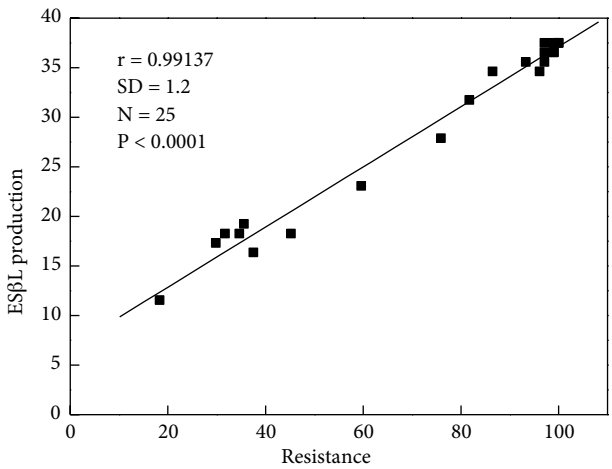

Figure 4. Correlation of antimicrobial resistant with ESβL production among 104 *P. aeruginosa* isolates. N: number of antimicrobial agents; r: Pearson's correlation coefficient.

in different sample sources. Comparable results showing variations in the incidence of *P. aeruginosa* among clinical samples have been reported (19–21). These variations may be due to environmental factors, nutritional requirements, or virulence factors.

Pathogenesis of *P. aeruginosa* is multifactorial, involving several virulence factors that include structural components, toxins, and enzymes (22). Some of the various virulence factors were selected in our study based on the importance of their role in disease production to establish the infection of *P. aeruginosa.* Extracellular enzymes are responsible for hemorrhage in internal organs in systemic disease, alter host cellular receptors, and alter microbial behavior by promoting invasiveness, serum resistance, and evasion of host immune mechanisms (23). In this study, *P. aeruginosa* isolates produced 3 enzymes (virulence factors), namely protease (85%), gelatinase (78%), and lipase (81%), suggesting that they were invasive isolates.

Rhamnolipids have also been associated with biofilm formation in *P. aeruginosa.* Davey et al. (24) demonstrated that the production of rhamnolipid surfactants influences the architecture of the formed biofilm. In this study, the majority (63.5%) of the isolates were rhamnolipid producers. This indicates that the producer isolates contributed to disease production in patients from whom clinical samples were taken.

Although biofilm formation (slime production) and CSH are 2 cell surface determinants that play significant roles in a wide range of microbial infections (25), a negative

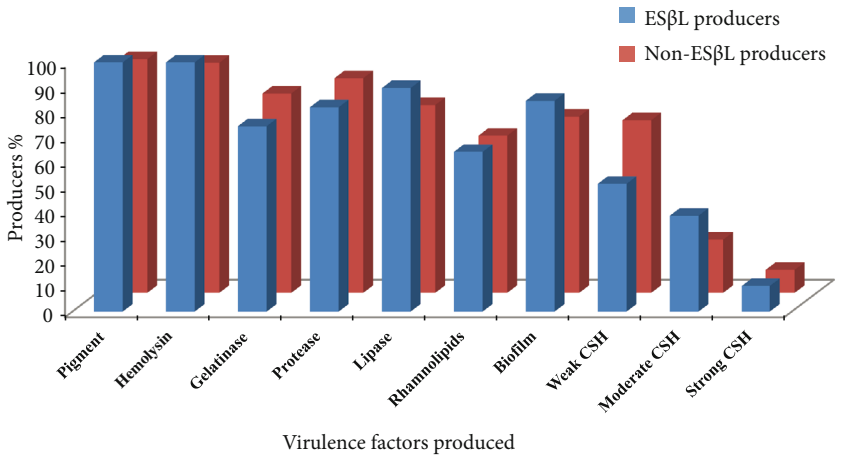

Figure 5. Virulence factors production in ESβL producers and non-ESβL producers among *P. aeruginosa* isolates.

association between biofilm formation and CSH was observed in our tested isolates. Thus, biofilm formation was probably strongly involved in the pathogenicity of the tested *P. aeruginosa* isolates.

One of the most worrisome characteristics of the tested *P. aeruginosa* was its low antibiotic susceptibility. This phenomenon of multiresistance of our isolates has previously been reported (26–28). The different antibiotic resistance patterns observed in the isolates indicate that the organism uses several mechanisms of resistance simultaneously, and that all isolates do not necessarily use the same mechanisms for resistance to particular classes of antibiotics. Furthermore, isolates that were resistant to 1 class of antibiotics were also resistant to at least 1 other class (29,30).

ESβLs, as a mechanism of antibiotic resistance, were phenotypically determined for all isolates (n = 39, 37.5%). The spread of ESβL-producing bacteria has become strikingly rapid worldwide, indicating that continuous monitoring systems and effective infection control measures are required (31–34).

The present study showed a significant correlation between ESβL producers and production of multiple virulence factors. A robust virulence factor repertoire may be essential for a pathogen to overcome intact host defenses, whereas it may be unnecessary in a compromised host, where antibiotic resistance may provide a substantial advantage to the survival of the pathogen. ESβL-negative isolates of *P. aeruginosa* produced multiple virulence factors, which supports the hypothesis that, although virulence factors and antibiotic resistance may confer increased fitness for human infections, they may do so via mutually exclusive pathways (35).

The emergence of ESβL-producing *P. aeruginosa* isolates with the expression of some virulence factors observed in this and other studies highlights how bacteria have evolved to overcome the pressures placed upon them by newer, more powerful antibiotics. They have had to rise to the challenges of these increasingly hostile environments in order to survive, and they have proven to be well adapted to the task. In severe infections, ESβLs and the expression of some virulence factors may work in harmony, resulting in a failure in treatment. Inadequate infection control measures have compounded this problem. Therefore, the regular detection of ESβLs by conventional methods should be carried out in every laboratory.

In conclusion, the data of this study have shown that ESβL-producing *P. aeruginosa* clinical isolates are armed with a large arsenal of virulence factors including pigment, hemolysin, lipase, protease, gelatinase, rhamnolipids, biofilm, and CSH. These factors could enable this pathogen to breach the human innate immune system, to intoxicate host cells, and to modulate human adaptive immune mechanisms, serving the goal of establishing systemic or more localized chronic colonization. The obtained results clearly indicate the importance of the recommendation that the antibiotic resistance as well as virulence factors of *P. aeruginosa* must be periodically studied in order to understand the possible coregulatory mechanisms that might be involved in their expression.

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