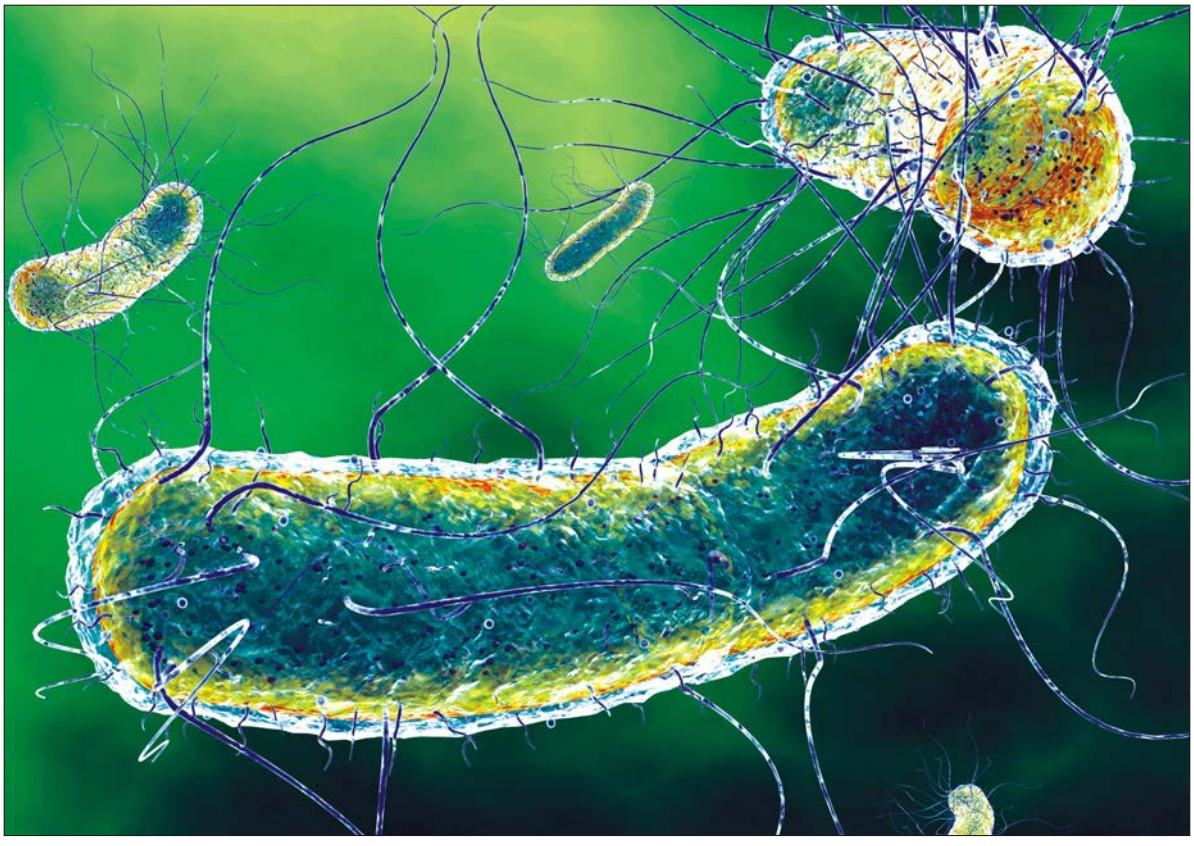

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Comparison of Virulence Factors among Clinical Isolates of *Pseudomonas aeruginosa* Producing and Non-producing Extended Spectrum β-lactamases

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ABSTRACT

Multidrug Resistance (MDR) *Pseudomonas aeruginosa* is an emerging nosocomial pathogen worldwide. In severe infections, Extended Spectrum β-lactamases (ESBLs) and expression of various virulence factors may work in harmony, resulting in the treatment failure. An association between ESBL production and two virulence factors: cell surface hydrophobicity and biofilm formation in *P. aeruginosa* has been demonstrated previously. This study was designed to compare the antimicrobial resistance pattern and production of some virulence factors in ESBL producers (ESBL+) and ESBL non-producers (ESBL-) clinical isolates of *P. aeruginosa*. ESBL production was detected by the double disk method and antimicrobial activity was tested by agar dilution methods. Antibacterial resistance pattern showed 48 different antibiotypes with 25 and 23 different types in ESBL+ and ESBL- isolates, respectively. The Minimum Inhibitory Concentrations (MICs) and percentage of isolates resistant to nearly all antibacterial agents tested, was significantly higher in ESBL+ isolates compared to ESBL-isolates ($p \leq 0.001$). The activity of LasA in ESBL+ isolates was higher than the negative isolates and statistically significant ($p \leq 0.05$). Expression of mannose sensitive haemagglutination and production of pyoverdin in the ESBL+ isolates was significantly lower than the ESBL-isolates ($p \leq 0.04$). No significant difference in the expression of mannose resistant haemagglutination and production of LasB was found between the ESBL+ positive or negative isolates. The result of present study suggests a correlation between ESBL phenotype and production of some factors that are reported to be involved in the virulence in *P. aeruginosa*.

Key words: *Pseudomonas aeruginosa,* virulence factor, antibiotic resistance, extended-spectrum β-lactamases(ESBLs), clinical samples

INTRODUCTION

Although, opportunistic in nature, *Pseudomonas aeruginosa* is now emerged as a major pathogen in patients with cystic fibrosis, weak or immunocompromised patients in the hospitals or nursing homes (Mesaros *et al*., 2007; Pearson *et al*., 1999). Multidrug resistance in *P. aeruginosa* is an increasing problem worldwide (Hocquet *et al*., 2006; Livermore and Woodford, 2006). Presence of low outer membrane permeability, expression of several efflux pumps and production of extracellular enzymes to inactivate antibiotics are the main reason of intrinsic resistance of *P. aeruginosa* to several antibacterial agents (Tomas *et al*., 2010; Abdi-Ali *et al*., 2007).

P. aeruginosa is able to form biofilm on different animate or inanimate surfaces; this property helps the survival and protects the bacteria from the immune system and antimicrobial agents inside the host (Deziel *et al.*, 2001; O'toole and Kolter, 1998). Slime layer production by some strains of *P. aeruginosa* can protect the bacteria and keep them away from the action of the antibacterial agents. Slime producing isolates are reported to have a high rate of resistance to antibacterial agents. Production of slime layer on medical devices is an important factor in pathogenesis of device associated infections with *P. aeruginosa* (Farrag, 2001).

Regulation of growth, production of many virulence factors and resistance to several antibacterial agents in *P. aeruginosa* is under the control of a population density, cell-to-cell communication called Quorum Sensing (QS) (Aendekerk *et al.*, 2005; Kong *et al.*, 2005; Ohman *et al.*, 1980). Expression of many virulence genes and acquisitions of genes involved in multidrug resistance are usually mediated by different efflux pumps and are under the control of QS (Deptula and Gospodarek, 2010). Different efflux pumps in *P. aeruginosa* are able to eject multiple antimicrobials from the cell, including β-lactamas and effects penicillins as well as non β-lactams such as fluoroquinolones (Livermore and Woodford, 2006). Other mechanism of resistance to β-lactamases is production of chromosomally encoded AmpC type β-lactamases and production of ESBLs (Hocquet *et al.*, 2006). ESBLs production is often associated with the mobile genetic elements, carrying genes that encode resistant to other antimicrobials such as aminoglycosides, sulfonamides, quinolones and trimethoprim (Livermore and Woodford, 2006; McGowan *et al.*, 1989). It has been shown that ESBLs producing isolates of *Klebsiella pneumoniae* are more invasive, producing more fimbrial adhesions and are more resistance to bactericidal activity of serum (Sahly *et al.*, 2004, 2008). This study was aimed to detect any possible correlation between the virulence factors and ESBL production in *P. aeruginosa* isolated from different clinical specimens of hospitalized patients.

MATERIALS AND METHODS

P. aeruginosa isolates were collected from different clinical samples of patients admitted to 3 different hospitals located in the southeast (Kerman) and 2 hospitals in the southwest (Shiraz) of Iran from January 2008 to June 2009. The isolates were identified by conventional biochemical tests (Hall, 2007). Only one isolate per patient was included. Type of sample and the place of the patient in the hospital were recorded. Totally 204 isolates were identified as *P. aeruginosa*. The isolated bacteria were kept at -70°C in TSB glycerol until use. After determination of Minimum Inhibitory Concentrations (MICs) of the isolates to 9 antimicrobial agents by agar dilution method (Hanlon *et al.*, 2007) the isolates showing resistance to 3 antibacterial agents from different classes were selected as the multiple drug resistance MDR. ESBL production in these isolates was determined by Combined Disc method (CD) as described previously (Norouzi *et al.*, 2010). In order to have a more diverse group of isolates, the isolates were selected based on antibiotypes and ESBL production. Fifty ESBL+isolates comprising of 25 antibiotypes and 50 ESBL-isolates, comprising of 23 antibiotypes were selected for the experiment.

For determination of Mannose Sensitive(MS) and Mannose Resistance (MR) haemagglutination pili the bacteria were serially sub cultured four times every 48 h in static brain heart infusion broth or nutrient broth for the detection of MS or MR, respectively (Evans *et al.*, 1979; Sahly *et al.*, 2008). Washed RBC prepared from freshly drawn guinea-pig blood or human blood group O^+, was used for the detection of MS or MR, respectively. The haemagglutination was graded from negative to 3+ according to Sahly *et al.* (2008).

For determination of Elastase (LasB) and Staphylolysin (LasA) activity the bacteria were grown up to the early stationary phase of growth (OD of 1.7 at 600 nm) in LB medium in a shaker incubator (120 rpm). LasB activity was determined with Elastin Congo red (ESR, Sigma, St. Louis, Mo, USA) as a substrate (Ohman *et al*., 1980). The insoluble Congo red was removed by centrifugation after 6 h incubation and the optical density of the mixture was determined at 495 nm. For determination of Las A activity the boiled cell pellet prepared from an overnight culture of *Staphylococcus aureus* (ATCC 25923) was used (Kong *et al*., 2005). The activity of LasA and LasB were presented as the change in the optical density of the mixture per hour per microgram of protein (Musthafa *et al*., 2010). Protein concentration in the bacterial cell free supernatants was determined by Bradford (1976).

Production of pyoverdin by the bacteria was determined on the cell free supernatant of the bacteria grown on LB medium with shaking (120 rpm-24 h). The optical density of the isolates was recorded at 600 nm. The relative concentration of pyoverdin in the cell free supernatant was determined at wavelength of 405 nm (Singh *et al*., 2010).

To detect any possible contribution between the virulence factors tested and the ESBL encoding plasmids, sixteen ESBL+ isolates were selected randomly. These isolates were sub cultured in LB broths containing 2% SDS at 42°C. The isolates were tested for the loss of the ESBLs phenotypes and by the decrease in MIC to cefotaxime and ceftazidime to susceptible level every 48 h until 60 days. After then 8 isolates which became ESBL$^-$ were tested again for the virulence factors mentioned.

Statistical analysis: Expression of different virulence factors in ESBL+ and ESBL- isolates were compared using chi-square analysis using SPSS software (SAS Institute, Cary, NC, USA) Data are expressed as Mean±Standard Division (SD). The correlation between the antibacterial resistance patterns and virulence factors with ESBL phenotype were tested by the independent sample t-test. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In present study resistance to some antibacterial agents was high and 71-72% of the isolates was resistance to tetracycline and trimethoprim /sulfamethoxazole (SXT), respectively. The high rate of resistance to these antimicrobials in gram negative bacteria are increasing worldwide (Manikandan *et al*., 2011; Shahcheraghi *et al*., 2009; Giske *et al*., 2008). The ESBL+ isolates were significantly more resistance to antibacterial agents compared with the ESBL-isolates ($p\leq0.001$), except for trimethoprim/sulfamethoxazole (SXT). SXT was the only antimicrobial agent that ESBL+isolate were more susceptible to it with ($p\leq0.04$). Nearly all the ESBL- isolates were found to be sensitive to ceftazidime (100%), ciprofloxacin (98%) and imipenem (98%) while for ESBL+isolates the resistance rate was significantly higher ($p\leq0.002$). The mean MIC for all the antibacterial agents tested (even for SXT) was statistically higher in the ESBL+compared to ESBL-isolates (Table 1). The same results has been reported for clinical isolates of enteric bacteria (Mansouri and Abbasi, 2010) and the gram negative bacteria from urinary tract infections (Selvakumar and Jasmine, 2007; Yousefi-Mashouf and Hashemi, 2006). This could be explained by the fact that ESBL+ bacteria usually had mobile genetic element coding gene for resistance to other antibacterial agent (McGowan *et al*., 1989). Statistically significant difference between the percentages of isolates resistance to antimicrobial agents in ESBL+ compared to non β-lactam antimicrobials is very important and ESBL production worked as a good marker for detection of MDR isolates. Bacterial isolates producing ESBL enzymes are increasing and they are among the most important problem contributing to significant mortality and morbidity in infected patients

Table 1: Comparison between antimicrobial susceptibility of 50 ESBL producers with 50 non-ESBL producer clinical isolates of *Pseudomonas aeruginosa*

	ESBL non producers		ESBL producers		
Antibacterial agents	Mean MIC±SD	% non susceptable	Mean MIC±SD	% non susceptable	*p-value
Cefotaxime	3.70±2.05	78	1.90±0.9	54	0
Ceftazidime	2.00±1.3	42	1.00±0.0	0	0
Ceftizoxime	4.80±1.8	94	2.18±0.9	32	0
Tetracycline	5.50±2.8	78	3.10±1.3	66	0
Gentamicin	4.60±3.2	60	1.70±1.5	24	0
Ciprofloxacin	3.02±1.9	56	1.00±0.3	2	0
Trimethoprime- sulfamethoxazole	4.80±2.8	66	3.90±1.8	76	0.04
Nalidixic acid	5.40±1.6	92	3.40±1.2	74	0
Imipenem	1.50±0.8	26	1.00±0.28	2	0.002

*p-values are related to the differences between the mean of MIC between the two groups of isolates, producing and non-producing ESBLs

(Ho *et al*., 2010). In the present study the most active agents against bacterial in both ESBL+and ESBL- were imipenem, ceftazidime and ciprofloxacin. In northwest of Iran (Urmia), the resistance rate of clinical isolates of *P. aeruginosa* to CIP and CAZ was reported to be 50 and 78% which is higher than this study. However, resistance to IPM in this study is much higher than Uremia (Jazani and Babazadeh, 2008). Imipenem is an antibiotic that fights serious infections caused by the bacteria. The current shift in the treatment of pseudomonad infections with this agent may be the cause for higher rate of resistance to it. Although, the most active agent in this study was IPM, simultaneous resistance of IPM resistance isolates to all other antibacterial agents tested is a matter of concern. Therefore, use of this agent should be restricted in order to avoid the emergence of extremely drug resistance isolates. Multidrug resistance in *P. aeruginosa* is increasing worldwide (McGowan *et al*., 1989). Since ESBL+ is very common in this area, therapy with compounds other than β-lactams such as polymyxins (polmyxinB, or colistin, polmyxin E) must be considered for the treatment of severally ill patients infected with these bacteria.

ESBL+ expressed lower percentage of mannose sensitive haemagglutination (MS) compared to ESBL-isolates. MSHA was detected for 44% of the ESBL+ and 26% of ESBL negative isolates and the difference was significance ($p \leq 0.04$). Moderate and strong type of reaction was also higher in these isolates (36%) compared with ESBL+ isolates (52%, $p \leq 0.04$) (Fig. 1). In this study all the isolates except one (a strain of *P. aeruginosa* with ESBL+phenotype isolated from a blood culture) expressed either MR or MS haemagglutination. These results are in accordance with the results obtained with *K. pneumoniae* and indicate that at least one fimbrial adhesion is required for colonization irrespective of the presence of ESBL plasmid (Sahly *et al*., 2008). *P. aeruginosa* isolates producing MS type haemagglutination are reported to be less hydrophobic than the organisms producing MR type haemagglutination (Sharma *et al*., 2007). Clinical isolates of *P. aeruginosa* producing ESBL are reported to be more hydrophobic and had a better ability to form biofilm compared to ESBL-isolates ($p \leq 0.01$) (Norouzi *et al*., 2010). The combination of these results suggests a correlation between production of these virulence factors and ESBL production.

Table 2 shows the difference between the activity of virulence factors in ESBL+ and ESBL- isolates. Mean activity of protease LasA was higher and LasB and pyoverdin production was lower in ESBL+ compared to ESBL-isolates ($p \leq 0.05$). The activity of Las A was lower in the control strain

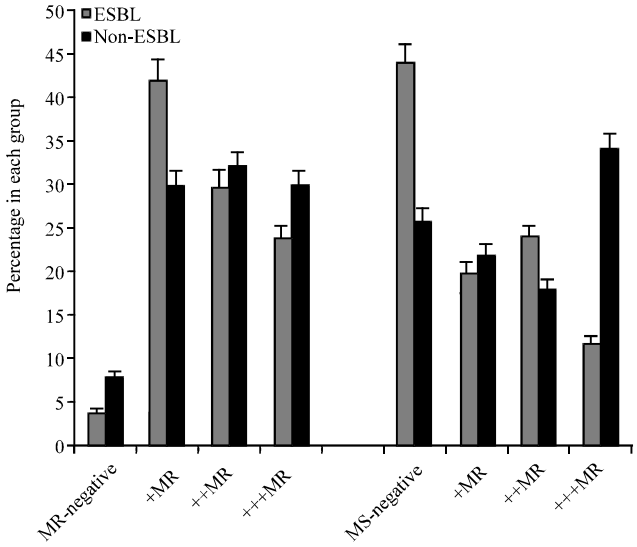

Fig. 1: Percentage of isolates producing of Mannose sensitive (MR) or Mannose resistance (MS) haemagglutination in ESBL producing (n = 50) and non-ESBL (n = 50) producing clinical isolates of *Pseudomonas aeruginosa*. Various degree of guinea-pig RBC (MS) or human O^+ RBC haemagglutination (MR) were graded from negative to 3+++

Table 2: Comparison between the activity of LasA protease, LasB elastase and pyoverdin in *Pseudomonas aeruginosa* clinical isolates producing (50 isolates, ESBL +) and non-producing ESBL (50 isolates, ESBL-) and the standard strains used as the control

[a]Virulence assay	ESBL+	ESBL-	p-value	[b]PAO1	[b]ATCC 27853
Las A, protease	9.5±7.48	7.29±2.45	0.05	5.77±1.23	8.15±1.04
Las B, elastase	5.4±4.19	7.12±5.28	0.074	7.11±1.02	2.87±0.78
Pyoverdin production	2893±1865	3635±242	0.018	4115.5±1123	1263.7±102

[a]All the assays were performed on cell free supernatant of bacteria grown in LB medium and are presented as the Mean±SD. [b]Control strains

PAO1 which was positive control for the experiments compared to standard strain ATCC 27853. Las B and pyoverdin production was higher in PAO1 compared to standard strain ATCC 27853. These results shows a similar activity of PAO1 strain and ESBL-isolates and strain ATCC 27853 with that of ESBL+ strains. In *P. aeruginosa* isolates with MDR phenotype the activity of LasB and pyocyanin or pyoverdin was reported to be lower than the susceptible isolates (Cowell *et al.*, 2003; Deptula and Gospodarek, 2010). LasA and LasB can decrease the level of toxins (ExoS, ExoU) and LasA/LasB plays a role in the regulation of the steady state level of ExoS and ExoT in the cell, modulating the effect of the bacteria to invade into mammalian cells (Cowell *et al.*, 2003). Our data showed that LasA/LasB in the ESBL+ isolates (2.2±0.97) was significantly higher than the ESBL-isolates (1.85±1.05; $p \leq 0.005$). It is possible that ESBL+ isolates regulates the production of these virulence factors in order to become invasive or cytotoxic in response to the environmental conditions.

No significant difference between productions of either or all of the virulence factors tested and the type of specimens from which the bacteria were isolated were found in this study. These results are similar to results obtained for *K. pneumoniae* and *Proteus* spp. (Mishra *et al.*, 2001; Sahly *et al.*, 2004, 2008). Plasmid curing experiments were performed to change the phenotype of

ESBL+ to ESBL-. ESBL negative isolates after curing had a reduced MIC against extended spectrum cephalosporins and became sensitive to these agents. However, Plasmid curing did not affects the virulence activity of the cured organisms significantly. These findings are in agreement with the results obtained with *K. pneumoniae* resistance to serum and suggests that the ESBL plasmids is unlikely to be involved in the expression of the virulence factors (Sahly *et al*., 2004).

An association between expression of certain virulence factor and different type of infections and MDR phenotype have been reported in *P. aeruginosa* (Bradbury *et al*., 2010; Deptula and Gospodarek, 2010) . In extra-intestinal *Escherichia coli* infections a significant association between ESBL phenotype and multiple virulence factors has been reported (Sharma *et al*., 2007). An association between high alginate production and higher susceptibility to antibacterial agents and lower β-lactamase production in *P. aeruginosa* isolates from cystic fibrosis patients has been documented (Ciofu, 2003). Expression of many virulence factors especially those involved in cell invasion such as colonial morphology, adhesion and aggregation in many gram negative bacteria including *P. aeruginosa* are regulated by phase variable mechanism (Deziel *et al*., 2001). Production of virulence factors could be also affected by the medium composition and environmental conditions. For example the production of rhamnolipid is reported to be higher in a medium containing an organic nitrogen source and high phosphate concentration (Mehdi *et al*., 2011). Although, this study was performed under similar growth conditions, testing the virulence factor under different conditions is recommended.

It is possible that *P. aeruginosa* clinical isolates producing ESBL regulate the expression of virulence factor by switching the production of certain factors on and off in special environment conditions in favor of their growth, survival and invasion into host tissue.

CONCLUSION

In conclusion, although the *in vivo* reaction of the bacteria or growth under different growth condition may affect the production of the virulence factors greatly, the *in vitro* experiments showed the more hydrophobic and stronger biofilm forming ESBL+isolates, were significantly more resistant to antibacterial agents then the ESBL-isolates. The ESBL+ isolates showed a higher activity for LasA but the production of pyoverdin, LasB and MS was significantly lower in these isolates compared to ESBL-isolates, suggesting the complexity of virulence factors expression under *in vitro* experiments. Study on the genes involved in the production of these factors and expression of them under different environmental condition may help to understand the possible co regulatory mechanisms which might be involved in the expression and their correlation with ESBL phenotype.

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