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# The Prevalence of Exoenzyme S Gene in Multidrug-Sensitive and Multidrug-Resistant *Pseudomonas aeruginosa* Clinical Strains

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Abstract

*Pseudomonas aeruginosa* rods are one of the most commonly isolated microorganisms from clinical specimens, usually responsible for nosocomial infections. Antibiotic-resistant *P. aeruginosa* strains may present reduced expression of virulence factors. This fact may be caused by appropriate genome management to adapt to changing conditions of the hospital environment. Virulence factors genes may be replaced by those crucial to survive, like antimicrobial resistance genes. The aim of this study was to evaluate, using PCR, the occurrence of exoenzyme S-coding gene (*exo*S) in two distinct groups of *P. aeruginosa* strains: 83 multidrug-sensitive (MDS) and 65 multidrug-resistant (MDR) isolates. *Exo*S gene was noted in 72 (48.7%) of the examined strains: 44 (53.0%) MDS and 28 (43.1%) MDR. The observed differences were not statistically significant (p = 0.1505). *P. aeruginosa* strains virulence is rather determined by the expression regulation of the possessed genes than the difference in genes frequency amongst strains with different antimicrobial susceptibility patterns.

Key words: *Pseudomonas aeruginosa*, exoenzyme S, multidrug sensitive, multidrug resistant, virulence genes

## Introduction

*Pseudomonas aeruginosa* is an opportunistic pathogen with increasing role in nosocomial outbreaks. It produces multiple virulence factors that have been implicated in both-pathogenesis and bacterial quorum sensing. *P. aeruginosa* strains virulence factors may be linked to bacterial cell surface or released outside the cells. Exoenzyme S is a secreted protein toxin with ADP-ribosyltransferase and GTP-ase activity (Barbieri and Sun, 2004). This toxin is encoded by *exo*S gene located at 4303141–4304502 chromosome position of *P. aeruginosa* PAO1 strain (Stover *et al.*, 2000). Exoenzyme S is formed by 453 amino acids and released in type-III secretion system directly to the cytosol of the animal epithelial cells (Barbieri, 2000; Krueger and Barbieri, 1995; Rumbaugh *et al.*, 1999b). Its main biological activity is the protein synthesis inhibition in eukaryotic cells. Exoenzyme S inhibits also phagocytosis of bacterial cells by macrophages. In this manner, it can interfere in host immunological response (Barbieri, 2000; Frithz-Lindsten *et al.*, 1997). It is also able to induce apoptosis of lymphocyte-T cells by their DNA fragmentation (Bruno *et al.*, 2000).

There are no direct criteria for *P. aeruginosa* strains classification into multidrug-resistant (MDR) group in the relevant literature (Falagas *et al.*, 2006; Magiorakos *et al.*, 2012). Many different authors usually provide their own definitions established for their studies purposes. According to the definitions formed in 1994 by the American Cystic Fibrosis Foundation, MDR strains should express resistance to all drugs from at least two different therapeutic groups, *e.g.* beta-lactams, aminoglycosides and quinolones. Such criterion was used by Hill *et al.* (2005). A different definition had been used by Tam *et al.* (2005). According to them, strains should be classified as MDR while resistance to ceftazidime, imipenem, tobramycin and ciprofloxacin can be found. Similar criteria were used by Obritsch *et al.* (2004) in the analysis of antimicrobial sensitivity of *P. aeruginosa* strains derived from the patients of the intensive care units, isolated in the United States between 1993 and 2002. Hsu *et al.* (2005) used both criteria – established by American Cystic Fibrosis Foundation, and their own. These authors had chosen a few indicators of antimicrobial resistance: piperacillin/tazobactam, ceftazidime and cefepime, imipenem, gentamicin, amikacin/tobramycin and ciprofloxacin/levofloxacin. Strains

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classified as MDR were resistant to at least two out of the antimicrobial groups mentioned above.

The aim of this study was to determine the *exo*S gene frequency and its difference in multidrug-sensitive (MDS) and MDR *P. aeruginosa* strains groups.

## Experimental

### Materials and Methods

A total of 148 non-duplicated *P. aeruginosa* clinical strains (83 MDS and 65 MDR) collected in the Department of Microbiology of dr. A. Jurasz University Hospital No 1 in Bydgoszcz were included in the study. The strains identification was done on the basis of standard microbiological procedures.

For antimicrobial susceptibility tests, using disc-diffusion method on Mueller-Hinton Agar (Becton Dickinson), the following antimicrobials were used: ticarcillin, piperacillin, ticarcillin/clavulanate, piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, amikacin, netilmicin, ciprofloxacin (Becton Dickinson). Results of the antimicrobial susceptibility tests were interpreted according to EUCAST Recommendations. *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains served as susceptibility testing quality controls. All the strains included into the study were, respectively, sensitive or resistant to all antimicrobials out of three groups: beta-lactams, aminoglycosides and fluoroquinolones and classified as MDS or MDR.

Bacterial genomic DNA was isolated with Genomic Mini kit (A&A Biotechnology), according to the manufacturer's protocol. In order to confirm the DNA isolation correctness and to avoid false-negative results all DNA samples were checked firstly: all of them were mixed separately with Loading Buffer DNA IV (AppliChem) and separated in 1% agarose gel (Bio-Rad) in 1 × TBE (Tris-Borate/EDTA, Bio-Rad) at 9 V/cm during one hour using MINI SUB™ DNA CELL (Bio-Rad) apparatus. After staining for 30 minutes in ethidium bromide solution and subsequent washing step for 20 minutes with deionized water, gels were visualized in UV light with Quantity One (Bio-Rad) program. DNA samples were then stored at 4°C until further use.

The prevalence of the exoenzyme S-encoding gene was determined by PCR. The amplification procedure was carried out according to Lanotte *et al.* (2004), in 0.2 ml test tubes (Eppendorf) in the final volume of 20 µl. *Taq* polymerase was used with the total activity of 1 U per sample in 1 × BD buffer, $MgCl_2$ at the final concentration of 1.5 mM (FirePol DNA Polymerase, Solis BioDyne) and dNTPs set at the final concentration of 200 µM (Solis BioDyne) were applied. Following primers were used: *exo*S F and *exo*S R, with sequences 5'→3': -CTTGAAGGGACTCGACAAGG- and -TTCA GGTCCGCGTAGTGAAT- (Integrated DNA Technologies), respectively, both at the final amount of 12.5 pmol per reaction. Isolated DNA samples were added subsequently. DNA isolated from *P. aeruginosa* PAO1 strain served as an amplification positive control. In the amplification procedure, thermal cycler GeneAmp® PCR System 2700 (Applied Biosystems) was applied the following conditions program: pre-amplification at 94°C for 3 minutes; amplification – 30 cycles, each consisting of: 94°C – 30 s, 53°C – 60 s, 72°C – 60 s; final elongation 72°C for 5 minutes. The obtained amplification products in the volume of 6 µl were mixed with Loading Buffer DNA IV (AppliChem) and separated in 1.5% agarose gel (Bio-Rad) in 1 × TBE (Bio-Rad), at 9 V/cm for 1.5 hour in MINI SUB™ DNA CELL (Bio-Rad) or SUB-CELL® GT (Bio-Rad). The 100–3000 bp DNA size marker (Solis BioDyne) was used. After staining for 30 minutes in ethidium bromide solution and subsequent washing for 20 minutes with deionized water, gels were visualized in UV light with Quantity One (Bio-Rad) system, photographed and stored. The gene identification was done on the basis of fragment size, as presented in Figure 1. Detection of a 504 bp product for the strain tested and the PAO1 control simultaneously was interpreted as a positive result.

Statistical analysis was performed using chi square test ($\chi^2$) with $\alpha \leq 0.05$ to determine the significance of the difference in *exo*S gene frequencies between MDS and MDR strains groups.

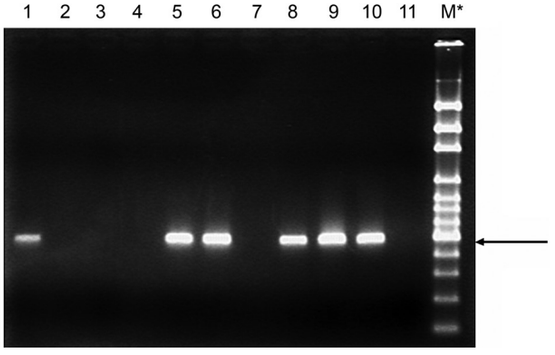


Fig. 1. An example of gel showing an electrophoretic separation of the *exo*S gene amplification product

* M-DNA size standard ranging from 100 to 3000 bp; 1–9 numbers of the examined strains; 10 – positive control; 11 – negative control; the arrow indicates the expected product size (504 bp).

## Results and Discussion

In the present work *exo*S gene was found in 72 (48.7%) of the investigated *P. aeruginosa* strains. Positive results were obtained for 44 (53.0%) of MDS and

28 (43.1%) of MDR tested strains. An example is shown in Figure 1. The difference in *exo*S gene prevalence between both examined groups was not statistically significant ($p = 0.1505$).

Since couple of years, there have been some information on reduced virulence of MDR *P. aeruginosa* strains available in the scientific literature (Deptuła and Gospodarek, 2010; di Martino *et al.*, 2002; Ramisse *et al.*, 2000, Khosravi *et al.*, 2016). One of the explanations of this phenomenon is that bacteria cells somehow selectively silence some genes and activate other ones, currently more important from the survival point of view. However, in the available literature there are only a few reports on comparison of virulence factors genes expression in *P. aeruginosa* strains sensitive and resistant to several antimicrobials groups (Linares *et al.*, 2005; Fuse *et al.*, 2012). Linares and co-workers (2005) observed reduction of the type-III secretion compounds in *P. aeruginosa* after overexpression of particular multidrug efflux pumps. Interestingly, on the basis of the Fuse *et al.* studies (2012), synthesis of another *P. aeruginosa* important virulence factor and pigment-pyocyanin is also reduced in MDR strains. Moreover, its synthesis also decreases after metallo-beta-lactamases genes transduction into non-MDR *P. aeruginosa* strains. These facts could explain in what manner the more resistant strains cause infections with the lowest frequency.

A second explanation for the reduced virulence of MDR strains is the appropriate bacterial genome management that allows for survival in the antibiotic-supplemented environment. In the literature numerous researchers characterize *P. aeruginosa* genetic features in terms of different conditions, *e.g.* origin, clinical specimen, hospitalization time. However, still none information on virulence genes frequency in MDS and MDR groups of *P. aeruginosa* strains can be found in the relevant literature.

There is a wide diversity in the prevalence of *P. aeruginosa* genetic features. In the available positions the highest percentage of *P. aeruginosa* strains carrying *exo*S gene was found in the studies conducted by Tingpej *et al.* (2007) and Idris *et al.* (2012), reaching 100% and 93.2%, respectively. In contrary, Azimi *et al.* (2016) had recently confirmed the *exo*S gene presence only in 26.3% of the examined strains which is the lowest value ever mentioned.

Lanotte *et al.* (2004) studies revealed *exo*S gene presence in a range of 64.7% up to 93.8% of the examined strains. The values were related to the clinical specimen type which *P. aeruginosa* strains were isolated from. Amongst the clinical strains the lowest percentage was observed for the strains isolated from urine. One of the highest values was found in sputum- and lung-derived isolates. It would suggest the crucial role of exoenzyme S presence in pulmonary infections.

According to the results obtained by Khosravi *et al.* (2016), *exo*S gene was noted in almost 86% of the strains derived from patients' burns while in the work of Wolska and Szweda (2009) the gene was observed in 75.8% of the strains tested. *Exo*S gene in the study mentioned above was noted with the lowest frequency amongst all of the examined strains when compared to other virulence factors genes. The study of *exo*S gene prevalence including the highest number of *P. aeruginosa* strains was carried out by Pirnay *et al.* (2009). They had investigated 328 unrelated *P. aeruginosa* strains isolated during 125 years, in 69 places from 30 countries in 5 continents. The *exo*S gene was present in 72.6% of the strains. Those strains were isolated not only from hospitalized patients, but also from animals and environmental samples. Results similar to those obtained by Pirnay *et al.* (2009), but exclusively for clinical *P. aeruginosa* strains, using PCR and also Southern hybridization for the first one, were found by Feltman *et al.* (2001) and Garey *et al.* (2008). The gene was present in 72% and 70.5% of the examined strains, respectively. According to Fazeli and Momtaz (2014) the *exo*S gene is one of the most common (67.64%) virulence gene found amongst the tested strains. Similar level of the *exo*S gene presence (65.4%) among the examined *P. aeruginosa* strains was also noted by Yousefi-Avarvand *et al.* (2015). Another study done by Zhuo *et al.* (2010) detected *exo*S gene in 65.1% of the examined *P. aeruginosa* strains collected from patients from five hospitals. Almost 64% of *P. aeruginosa* isolates from ocular infections, examined by Choy *et al.* (2008) were also positive for *exo*S gene. Mitov and co-workers (2010) showed the presence of *exo*S gene amongst 62.4% of the examined *P. aeruginosa* strains but they did not confirm differences in the spread of the gene neither amongst MDR, nor non-MDR strains population. In the work published by Amirmozafari *et al.* (2016) the frequency of *exo*S gene reached 61% but none association was found between strains resistance and gene presence. The results demonstrated by Finlayson and Brown (2011) show that *exo*S genes presence is observed in approximately half of the examined strains while Winstanley *et al.* (2005) has confirmed *exo*S gene presence only in 38% of *P. aeruginosa* strains that had been examined. These latter values seems to be really close to the results obtained in this study where *exo*S gene was present in 43.1% up to 53.0% of the examined non-duplicated MDR and MDS strains, respectively.

Noteworthy, in the available literature, there is spare information on reduced *exo*S genes carriage amongst MDR *P. aeruginosa* strains to compare to our results. The only paper that includes similar research approach (Khosravi *et al.*, 2016) shows limited *exo*S gene presence in MDR comparing to overall strains population (77.1% *vs.* 85.8%) derived from burn patients.

Although, the aim of this study was only to estimate the *exo*S gene carriage, not the ability of exoenzyme S synthesis, it is also very interesting issue. Zhang and Wei (2009) study proved that the expression of virulence factors in *P. aeruginosa* strains is a very complex process. Exoenzyme S synthesis level may be for example raised by the glutathione concentration. Noteworthy, exoenzyme S is not always expressed, Tartor and El-Naenaeey (2016) detected its synthesis amongst 78.6% of *P. aeruginosa* strains. Similarly, results obtained by Tingpej *et al.* (2007) revealed that only 77% of the *exo*S gene-carrying strains synthesized this toxin in the respiratory tract infections studies. Meanwhile, during infection exoenzyme S production is necessary to invade *e.g.* epithelial cells, what is achieved by its injection directly into human cells (Heimer *et al.*, 2013; Hayashi *et al.*, 2015).

Joly *et al.* (2005), using RT-PCR, indicated increased *exo*S expression at the beginning of experimental *P. aeruginosa pneumonia* in rat model. The highest exoenzyme S transcript levels were present during the first two days of the infection. Its presence was linked with 29% mortality and, after two days, a drop in its synthesis was observed. Similar correlation in the animal model of *exo*S expression was indicated by Pierre *et al.* (2008). These authors claim that the expression of all genes connected with type-III secretion system decreases during chronic disease. In contrary to this conclusion, Hamood *et al.* (1996) and Rumbaugh *et al.* (1999a) claim that prolonged *P. aeruginosa* infection seems to increase exoenzyme S production. Moreover, it is synthesized significantly more often by wound- and urinary tract infections-derived *P. aeruginosa* strains (Hamood *et al.*, 1996).

Taken together, the results of this study indicate for the first time, although not statistically important, reduced exoenzyme S genes carriage in MDR *P. aeruginosa* strains when compared to MDS. All the facts mentioned above prove the advanced nature of *exo*S gene carriage, as well as expression, and indicate that further studies on that issue are necessary.

## Conclusions

Exoenzyme S gene is not present in all *P. aeruginosa* strains, therefore not all of the strains have the ability to synthesize this virulence factor.

*Exo*S gene is noted more often amongst multidrug-sensitive *P. aeruginosa* strains, when compared to multidrug-resistant ones, but no statistically significant difference was observed.

The reduced virulence of multidrug-resistant *P. aeruginosa* strains is more likely caused by gene expression regulation, and not by the absence of virulence genes.

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