**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 *exoS* gene located at 4303141–4304502 chromosome position of *P. aeruginosa* PAO1 strain (Stover et al., 2000). ExoS enzyme S is formed by 453 amino acids and released in type-III secretion system directly to the cytosol of the animal epithelial cells (Barbieri and Sun, 2004). This toxin is encoded by *exoS* gene located at 4303141–4304502 chromosome position of *P. aeruginosa* PAO1 strain (Stover et al., 2000). 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, cefazidime and ceftazidime, imipenem, gentamicin, amikacin/tobramycin and ciprofloxacin/levofloxacin. Strains
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, MgCl₂ 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: exoS F and exoS R, with sequences 5’→3’: -CTTGAAGGGACTCGACAAGG- and -TTCA GGTCGGGTAGTGAAT- (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 PA01 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 correspondently. DNA isolated from P. aeruginosa PA01 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 (χ²) with α ≤ 0.05 to determine the significance of the difference in exoS gene frequencies between MDS and MDR strains groups.
28 (43.1%) of MDR tested strains. An example is shown in Figure 1. The difference in exoS 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 (Deptaula 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 exoS 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 exoS gene presence only in 26.3% of the examined strains which is the lowest value ever mentioned.

Lanotte et al. (2004) studies revealed exoS 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), exoS gene was noted in almost 86% of the strains derived from patients’ burns while in the work of Wolska and Szveda (2009) the gene was observed in 75.8% of the strains tested. ExoS 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 exoS 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 exoS 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 Montaz (2014) the exoS gene is one of the most common (67.64%) virulence gene found amongst the tested strains. Similar level of the exoS 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 exoS 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 exoS gene. Mitov and co-workers (2010) showed the presence of exoS 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 exoS gene reached 61% but none association was found between strains resistance and gene presence. The results demonstrated by Finlayson and Brown (2011) show that exoS genes presence is observed in approximately half of the examined strains while Winstanley et al. (2005) has confirmed exoS 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 exoS 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 exoS 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 exoS 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 exoS gene carriage, not the ability of exoenzyme S synthesis, it is also very interesting issue. Zheng 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-Neaaya (2016) detected its synthesis amongst 78.6% of *P. aeruginosa* strains. Similarly, results obtained by Tingnej et al. (2007) revealed that only 77% of the exoS 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 exoS 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 exoS 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 exoS 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. ExoS 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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**Literature**


