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FENOTIPSKA SVOJSTVA I REZISTENCIJA NA ANTIBIOTIKE SOJEVA BAKTERIJE *RALSTONIA PICKETTII* IZ SUSTAVA ZA ULTRAČISTU VODU

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PHENOTYPIC PROPERTIES AND ANTIBIOTIC RESISTANCE OF *RALSTONIA PICKETTII* STRAINS FROM HIGH-PURITY WATER SYSTEMS

DOCTORAL THESIS

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SAŽETAK

Gram-negativna bakterija *Ralstonia pickettii* stvara biofilm u farmaceutskim sustavima za ultračistu i pročišćenu vodu. U slučaju neučinkovite sterilizacije može kontaminirati sterilne otopine za parenteralnu primjenu putem kojih ulazi u bolnički sustav i kolonizira bolnički okoliš i pacijente. Studije o antimikrobnoj osjetljivosti i sustavnom praćenju *R. pickettii* u bolničkom okruženju vrlo su ograničene, posebice na većem broju izolata. Jednako, nevelik je broj istraživanja okolišnih izolata *R. pickettii* uz nestandardizirano testiranje osjetljivosti na antibiotike. Ovo ispitivanje provedeno je s ciljem utvrđivanja svojstava i osjetljivosti na antibiotike izolata *R. pickettii* s područja Republike Hrvatske. U petogodišnjem periodu prikupljen je 81 izolat iz različitih industrijskih sustava za farmaceutsku čistu i ultračistu vodu i laboratorijsku pročišćenu vodu. Osjetljivost na antibiotike ispitana je E-testom i disk difuzijskim testom. Lančanom reakcijom polimeraze ispitano je prisustvo gena *bla*$_{OXA22}$ i *bla*$_{OXA60}$ za oksacilinaze, gena *bla*$_{IMP}$, *bla*$_{VIM}$, i *bla*$_{NDM}$ za metalo-β-laktamazu, gena *bla*$_{OXA48}$ za hidrolizirajuću karbapenemazu, gena *armA, rmtA, rmtB, rmtD* za metil-transferaze i gena *aac(3')-II, aac(6')-Ib, aph(3')-Ia, ant(2'')* za aminoglikozid-modificirajuće enzyme. Ispitivanje prijenosa gena rezistencije na meropenem i gentamicin s *R. pickettii* na primatelja *E. coli* J53 koja je bila osjetljiva na ta dva antibiotika, a rezistentna na natrijev azid provedeno je metodom konjugacije. Izolati su genotipizirani gel elektroforezom u pulsirajućem polju. Dobiveni rezultati su otkrili različite profile osjetljivosti/rtezistencije. Većina izolata bila je otporna na kolistin, aztreonam, ertapenem, aminoglikozide i meropenem. Visoke stope rezistencije uočene su i kod tikarcilin/klavulanske kiseline, tikarcilina, amoksicilin/klavulanske kiseline i ampicilina. S druge strane ispitivani izolati su pokazali visoke stope osjetljivosti na tetracikline, tigeciklin, trimetoprim-sulfametoksazol, imipenem, cefepime, cefaleksin, cefoksitin, cefotaksim, piperacilin-tazobaktam, ciprofloksacin, ceftazidime i piperacilin. Modificirani Hodge test bio je pozitivan u 51,9% izolata, što bi upućivalo na stvaranje karbapenemaza. Gen *bla*$_{OXA22}$ je otkriven u 37,0% izolata, a gen *bla*$_{OXA60}$ u 80,3% izolata. Tako visoka prisutnost gena *bla*$_{OXA22}$ i *bla*$_{OXA60}$ ukazuje kako "sterilna" voda može predstavljati golemi okolišni rezervoar gena rezistencije koji se, onda primjenom iste, mogu prenijeti u kliničko okruženje i predstavljati prijetnju uspješnom liječenju infekcija. Nisu detektirani geni za metalo-β-laktamaze niti za karbapenemaze kao ni geni za najčešće 16SrRNA metil-transferaze niti za aminoglikozid modificirajuće enzime. U izolatima je dokazano prisustvo endotoksina. Dokazana je pokretljivost plivanja i trzanja, proizvodnja izvan stanične polimerne supstance te sposobnost proizvodnje biofilma. Gel elektroforeza s pulsirajućim poljem pokazala je veliku srodnost izolata i identificirana su tri glavna klastera koji sadrže podklastere. *R. pickettii* treba

shvatiti ozbiljno kao mogućeg uzročnika nozokomijalnih infekcija kako bi se osigurala odgovarajuća terapija, spriječio razvoj rezistentnih sojeva i pokušala smanjiti mogućnost njezinog preživljavanja u sustavima za čistu, ultra čistu i laboratorijsku pročišćenu vodu.

Ključne riječi: *Ralstonia pickettii*, osjetljivost na antibiotike, *bla*$_{\text{OXA22}}$ i *bla*$_{\text{OXA60,}}$ *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, i *bla*$_{\text{NDM,}}$ *bla*$_{\text{OXA48,}}$ *armA, rmtA, rmtB, rmtD, aac(3')-II, aac(6')-Ib, aph(3')-Ia, ant(2''),* pokretljivost, biofilm, endotoksin.

SUMMARY

Introduction: Gram-negative bacterium *Ralstonia pickettii* is often isolated from the various areas: from industrial water production systems, soil, animals, plants, and from the oral cavity and upper respiratory tract as part of the commensal flora. It is isolated very often from pharmaceutical water systems where the concentration of nutrients (carbon) is low. It is oligotrophic and, in these conditions, despite low amounts of nutrients, it forms a biofilm. Further reproduction and production of the biofilm takes place at the expense of the dead cells in the biofilm, which, by lysing, release many substances that can be a nutrients source for further cell growth. It colonizes the hospital environment and patients. Therefore, in immunocompromised and seriously ill persons it can cause serious infections, often resistant to antibiotic treatment. *R. picketti* is an opportunistic pathogen of low virulence. It rarely causes infections in healthy individuals but can have high clinical significance. The aim of this research was to study phenotypic properties, sensitivity to antibiotics, detection of resistance genes, and the relatedness of tested isolates collected in a five-year period from different areas of Croatia.

Materials and methods: Eighty-one (81) *Ralstonia pickettii* isolates were obtained during a five-year period (2011-2015), from two different areas in Croatia (area I and area II) and from different sources: two pharmaceutical-industrial ultra-pure water (UPW) systems and three laboratory purified water (LPW) systems. Bacterial isolation from water samples was carried out by the membrane filtration method. The samples were filtered out by 0.45 μm membrane filters, which were then placed on the surface of R_2A and Tryptic soy agar and cultured for 24 hours at 37 °C. Grown colonies that indicated a possible *R. pickettii* were additionally identified based on the microscopic appearance of the cells, and by biochemical tests. All 81 isolates were subsequently confirmed as *R. pickettii* by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and by polymerase chain reaction (PCR) using species specific primers. The reference strain *R. pickettii* ATCC 27511 was used as a control strain.

Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion test (DD) on Mueller Hinton agar (MHA) for 26 antibiotics with antimicrobial discs. E-test was performed to determine minimum inhibitory concentrations (MIC) against 12 antibiotics, ceftazidime, cefepime, ceftriaxone, cefotaxime, imipenem, meropenem, piperacillin-tazobactam, tobramycin, gentamicin, amikacin, netilmicin and ciprofloxacin. Zone diameter and breakpoints were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antibacterial Susceptibility Testing (EUCAST).

Production of carbapenemases, metallo-β-lactamases, and extended spectrum β-lactamases (ESBL) were assessed phenotypically; modified Hodge test, carbapenem inactivation method (CIM), combined disk test **(**CDT). The presence of genes *bla*$_{OXA22}$ and *bla*$_{OXA60}$ for oxacillinases, genes *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{NDM}$ for metallo-β-lactamases, gene *bla*$_{OXA48}$ for hydrolyzing carbapenemase, genes *armA*, *rmtA*, *rmtB*, *rmtD* for methyl-transferases and the genes *aac(3')-II*, *aac(6')-Ib*, *aph(3')-Ia*, *ant(2'')* for aminoglycoside-modifying enzymes was tested by PCR. Transfer of resistance to meropenem and gentamicin was tested by the broth conjugation method using *E. coli* J53 resistant to sodium azide as a recipient, but sensitive to gentamicin and meropenem. Isolates were genotyped by Pulsed-Field Gel Electrophoresis (PFGE). Endotoxin production was tested with Limulus Amebocyte Lysate (LAL) test. The production of extracellular polymer substance (EPS) by *R. pickettii* was tested on different materials. Biofilm production was investigated by microtiter plate biofilm assay. Motility was tested on nutrient media in a test tube and in Petri dishes with different agar consistencies of 0.3%-1.5%.

Results: Eighty-one (81) isolates collected from pharmaceutical and laboratory water production systems from two different areas of Croatia were phenotypically and biochemically identified as *R. pickettii* and confirmed by MALDI-TOF and PCR.

The results of *R. pickettii* antibiotic susceptibility testing using the disk diffusion method were as follows: high resistance rates were obtained for colistin (100%), aztreonam (96.3%), ertapenem (91.3%), and all aminoglycosides: netilmicin (88.9%), amikacin and tobramycin (87.7%), and gentamicin (86.4%). High resistance rates were also observed with beta-lactam antibiotics of the penicillin group: amoxicillin/clavulanic acid (71.6%), ticarcillin (67.9%), ticarcillin/clavulanic acid (61.7%), and ampicillin (58.0%). Significantly different resistance rates were obtained for piperacillin and piperacillin/tazobactam. Almost all isolates were susceptible to piperacillin and piperacillin/tazobactam, only 2 isolates (2.5%) were resistant, both from area II, label K (K82-1 and K82-2). All 81 isolates (100%) were sensitive to cephalosporin antibiotics ceftriaxone, cefotaxime, cefepime, and most to cephalexin (96.3%), cefoxitin (97.5%) and ceftazidime (81.5%). All 81 isolates (100%) were susceptible to imipenem, tigecycline, tetracycline and trimethoprim-sulfamethoxazole, and almost all to ciprofloxacin (93.8%). Resistance to meropenem was recorded in 29.6% of isolates, while 40.7% of isolates were intermediately sensitive to meropenem. Only 8,6% of the isolates were resistant to chloramphenicol, 40.7% of the isolates were moderately susceptible to chloramphenicol, while half of the isolates (50,6%) were susceptible.

In the E-test, all 81 isolates (100%) were susceptible to piperacillin/tazobactam, ceftriaxone and cefotaxime, 96.3% were susceptible to ciprofloxacin, and three (3.7%) were intermediately susceptible to ciprofloxacin. 71.6% (58/81) of the isolates were sensitive to imipenem, while 28.4% (23/82) were intermediately sensitive. 67.9% (55/81) of the isolates were sensitive to ceftazidime and 66.7% (54/81) to cefepime. The E-test obtained a high resistance rate to meropenem (95.1%), and aminoglycosides, 85.2% for gentamicin and amikacin each, 86.4% for tobramycin, and 87.7% for netilmicin. Resistance to meropenem obtained in the E-test (95.1%) is significantly higher than the one obtained by the disk diffusion method (29.6%).

Resistance to all four tested aminoglycoside antibiotics was almost uniform. The most frequent phenotype of aminoglycoside resistance was T-G-A-N (tobramycin, gentamicin, amikacin, netilmicin) and was exhibited by 85.2% of the isolates (69/81), and they all were from the same area (area I), from different sources. Only two isolates from the same area, HL28 and HL30, were sensitive to tobramycin, amikacin, and gentamicin and resistant only to netilmicin (phenotype N). On the other hand, all 10 isolates from area II showed the opposite results; eight isolates were sensitive to all four aminoglycoside antibiotics; one isolate was sensitive to tobramycin and netilmicin and intermediately sensitive to gentamicin and amikacin, and one was resistant only to tobramycin and sensitive to the other three aminoglycosides.

No *R. pickettii* isolate showed the production of extended spectrum β-lactamase in the double disc test.

The modified Hodge test was positive in 51.9% (42/81) of isolates, weakly positive in 25.9% (21/81) and negative in 22.2% (18/81) of isolates. All 9 CIM tested isolates were positive.

The CDT was positive in 97.5% isolates (79/81) when meropenem was applied as an indicator disk, whereas 95.1% isolates were negative with imipenem (77/81). Four isolates (4.9%) exhibited positive CDT with both carbapenems. Despite the positive results of the Hodge and CDT tests in 28 isolates, gene encoding carbapenemases (*bla*$_{OXA48}$) and genes encoding metallo-β-lactamases (*bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{NDM}$) were not detected by molecular testing. The *bla*$_{OXA60}$ gene was detected in 80.3% (65/81) whereas the *bla*$_{OXA22}$ gene was detected in 37.0% (30/81) isolates. All isolates that tested positive for *bla*$_{OXA22}$ were positive for *bla*$_{OXA60}$ as well. Genes *armA, rmtA, rmtB, rmtD* for methyl-transferases and the genes *aac(3')-II, aac(6')-Ib, aph(3')-Ia, ant(2'')* for aminoglycoside-modifying enzymes were not detected by PCR.

All tested isolates (81) demonstrated twitching motility on a solid nutrient medium with 1% agar. Swimming motility on semi-solid substrates with 0.3% agar was also demonstrated for all tested isolates both in the test tube and in the Petri dish. Swarming was not proven in any isolate. In this study, the production of EPS in *R. pickettii* was demonstrated. Production of

biofilm was confirmed in 37% (30/81) isolates. In this study, it was proven that *R. pickettii* possesses an endotoxin that is thermostable and is released by destroying the bacterial cell at a high temperature of 136 °C. PFGE revealed three larger clones, each containing subclones that differed in one to three bands.

Conclusion: The results obtained in this study characterize environmental bacterium *R. pickettii* isolated from water systems in Croatia. Its antibiotic resistance, ability to produce EPS and biofilm, types of motilities, ability to produce endotoxin, and finally the relatedness of isolates from different sources from different areas of Croatia were examined.

The antibiotic resistance results of this study are in line with previously published results, the most of isolates were susceptible to most of the tested antibiotics, except for colistin, aminoglycosides, ertapenem, aztreonam and meropenem.

The isolates from different locations clustered together, pointing out to a possible common source. Further comparison with currently unavailable local clinical isolates would be necessary to analyse the driving force for the development of relevant pathogens from typically environmental harmless bacteria. *R. pickettii* should be taken seriously as a possible cause of nosocomial infections to ensure adequate therapy, to prevent the development of resistant strains and to reduce the possibility of *R. pickettii* surviving in clean and ultra clean water systems.

These are extremely valuable data, and it would be useful to collect clinical isolates and test their resistance and compare with the obtained results. It might help in monitoring the change of an environmental bacterium into a potential pathogenic microorganism. It would be equally interesting to conduct a study in which a possible connection of some metabolic problems and conditions with the presence of *R. pickettii* in digestion would be established, because it was observed that *R. pickettii* appears more often in some metabolic conditions (e.g., diabetes, obesity). It would be useful to study biofilm production on different systems, with constant water flow to mimic the aqueous environment of a pharmaceutical water system. Such a test could be additionally carried out with the application of disinfection and sanitation at certain time intervals to determine the optimal removal of biofilm and extend the time of repeated washing and disinfection/sanitization of the system.