Title: The antibiotic susceptibility of water based nosocomial pathogens *Ralstonia pickettii* and *Ralstonia insidiosa*.

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Abstract

Ralstonia pickettii and *Ralstonia insidiosa* are waterborne bacteria that can survive and grow in various water sources that are emerging pathogens in hospital settings. Not much is known about the antibiotic resistance of these bacteria. Previous reports of antimicrobial susceptibility have been largely limited to a few clinical strains with no accounting for genotypic or phenotypic diversity or that these species could vary from the set breakpoints. E-tests and disc diffusion tests were carried out to compare the antimicrobial susceptibilities to twelve different antibiotics of sixty-eight different isolates of *R. pickettii* (fifty-three) and *R. insidiosa* (fifteen) from varying environments, which have previously been well characterised both phenotypically and genetically. The majority of the *R. pickettii* and *R. insidiosa* isolates showed susceptibility to most of the antibiotics tested in this study. The most effective were found to be the quinolones and sulphamethoxazole/trimethoprim. Antibiotic susceptibility was also found not vary between environmental niches for *R. pickettii* and *R. insidiosa* isolates.

Introduction

Ralstonia pickettii is abundant in the environment having been isolated from a wide array of environmental sources (Ryan *et al*., 2011c). The bacterium has been recovered from numerous water sources including municipal drinking water supplies (Lee *et al.,* 2010), bottled water (Falcone-Dias *et al.,* 2012), dental water supplies (Szymańska 2006), hospital water supplies (Kendirli *et al.,* 2004; Ryan *et al.,* 2006), space shuttle water systems (Koenig and Pierson 1997), standard purified water (Penna *et al.,* 2002), laboratory based high-purity water systems (Adley *et al.,* 2005) and industrial Ultra-pure/High Purity water (Kulakov *et al.,* 2002; Adley and Saieb 2005; Bohus *et al.,* 2010). *R. pickettii* has also been identified in forming and maintaining biofilm in plastic industrial water piping (Anderson *et al.,* 1990; Adley and Saieb 2005). The bacterium has also been found in a wide variety of clinical environments worldwide and has become recognised as a nosocomial pathogen that is particularly associated with patients who are immunosuppressed or are in some other way debilitated (Ryan *et al.,* 2006). Conditions associated with *R. pickettii* range from range from minor infections to more severe invasive infections such, sepsis or meningitis. Minor respiratory illnesses were found in 34 patients in an outbreak due to contaminated solutions (Labarca *et al*., 1998). More invasive infections such as osteomyelitis (Wertheim and Markovitz 1992) have been reported in a 71 year old man suffering from chronic renal failure, a 75 year old women in Bulgaria was also with renal failure was reported to have a case of *R. pickettii* related renal failure sepsis (Strateva *et al*., 2012). Cases of meningitis have also been reported associated with *R. pickettii* including that of a 54 year old male who was otherwise healthy (Heagney 1998). These infections have been recorded in association with contamination of hospital water supplies such as respiratory solutions (water based) and Water For

Injection (WFI) (Gardner and Shulman, 1984; McNeil *et al.,* 1985; Roberts *et al.,* 1990; Maki *et al*., 1991; Raveh *et al*., 1991; Labarca *et al*., 1998). The majority of clinical isolates of *R. pickettii* are multiresistance to common antibiotics (Zellweger *et al.,* 2004). The bacterium has shown itself to be very resilient to treatment in water supplies with resistance to disinfectants such as chlorhexidine and under certain circumstances *R. pickettii* has been shown to penetrate 0.2 micron filters (Sundaram *et al*., 1999, 2002; Adley *et al.,* 2005)

R. pickettii has been shown to survive in low nutrient (oligotrophic) conditions (McAlister *et al.,* 2002). In addition, the bacterium has been shown to possess a wide range of biodegradative abilities that could be used for commercial applications (pollution clear ups) and that could assist in the survival and adaption of the organism to low nutrient environments (Ryan *et al.,* 2007). Integrating Conjugative Elements-like (ICE) elements (Such as Tn*4371*) have been found in various isolates of this bacterium signifying a degree of plasticity in their genomes (Ryan *et al.,* 2009, Van Houdt *et al*., 2012).

Ralstonia insidiosa is the most closely related bacteria to *R. pickettii* (Ryan *et al.,* 2011a) and has also been isolated from natural water sources such as ponds and rivers, soil, activated sludge (Coenye *et al.* 2003), laboratory purified water systems (Ryan *et al*., 2011b), and industrial Ultra-pure/High Purity water (Ryan *et al.,* 2011b) and water distribution systems (Hoefel *et al.,* 2005). It has been reported to be the causative agent of hospital based infections in two immunocompromised individuals (Van der Beek *et al.,* 2005) and was found to be the cause of sepsis in eight hemodialysis patients in the Czech Republic (Orlíková *et al.,* 2012) due to contaminated hemodialysis solutions. Both bacteria have been found in the lungs of cystic fibrosis sufferers (Coenye *et al.,* 2003).

The treatment of *R. pickettii* infections is often challenging as this bacterium has been reported as being intrinsically resistance to many antimicrobial agents (Zellweger *et al*., 2004; Ryan *et al.,* 2006) which could be due to the presence of mobile genetic elements (Ryan *et al.,* 2009). The antimicrobial resistance mechanisms in *Ralstonia* sp. are generally unknown; however, two specific mechanisms of resistance to some β-lactam antibiotics have been identified. Two different types of class D β-lactamases have been identified: *bla*$_{\text{OXA-22}}$ and *bla*$_{\text{OXA-60}}$ and these genes have contributed to the multidrug-resistant phenotype displayed by *R. pickettii*. *bla*$_{\text{OXA-22}}$ has activity against to benzylpenicillin, cloxacillin, and restricted-spectrum cephalosporins and *bla*$_{\text{OXA-60}}$ has activity against imipenem (Nordmann *et al.,* 2000; Girlich *et al.,* 2004).

R. pickettii and *R. insidiosa* are a growing threat in clinical situations, this is mainly due to their presence in water and their ability to survive in purified/distilled water used to make-up medicinal products in hospitals for patient treatment, their ability to survive in nutrient limited environments and to survive treatment with common clinical antimicrobial agents such as chlorhexidine (Adley *et al.,* 2005; Ryan *et al.*, 2006, 2011c). It is thought that infection with *Ralstonia* spp. is mainly due to environmental sources such as contaminated water supplies (Ryan *et al.,* 2006), similar to *Brevundimonas diminuta* (Han *et al.,* 2005).

There is limited information on the surveillance and monitoring of the antibiotic resistance of these bacteria and even less information on any possible differences in antibiotic resistance between clinical and environmental isolates. This study, which used a unique collection of *R. pickettii* and *R. insidiosa* (sixty-eight isolates in total) which included clinical and environmental isolates, reports the antimicrobial susceptibility profile of *R. pickettii* and *R. insidiosa* isolates as well as to compare the

Etest and disk diffusion methodologies for determining the antimicrobial susceptibility of these bacteria.

Methods

R. pickettii and R. insidiosa strains

Sixty-eight isolates of a unique culture collection including fifty-three *R. pickettii* and fifteen *R. insidiosa* were examined; thirty-two isolates came from industrial purified water, eleven from various laboratory purified water sources, eight from various clinical sources, six were isolated from washing water from an Endoscopy unit, two (whole genome sequenced isolates) from a heavy metal contaminated lake, two purchased soil strains, five purchased strains of *R. pickettii* and two purchases strains of *R. insidiosa* were from various sources including the BCCM/LMG Bacteria Collection (Ghent, Belgium), the Japan Collection of Microorganisms (Hirosawa, Wako-shi, Japan); the National Collection of Type Cultures (London, UK); the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany); the Collection Bactèrienne de l' Institut Pasteur (Paris, France), the American Type Culture Collection (Manassas, Virginia, USA)and the Culture Collection University of Göteborg, (Göteborg, Sweden). The full list can be seen in Table 1. The isolation, identification and genotyping of all these isolates is dealt with in Ryan *et al.,* 2011b. Briefly these isolates were identified using a combination of biochemical and PCR based method of identification. Genotyping was carried out using a whole genome approach with RAPD and BOX PCR (Ryan *et al.,* 2011b.). All isolates were stored at -80°C in Nutrient Broth (Oxoid, Basingstoke, UK) with 50% glycerol. Isolates were grown aerobically on Nutrient Agar (Oxoid, UK) and incubated overnight at 30°C.

Antimicrobial susceptibility disc diffusion tests

The antibiotic discs were purchased from Oxoid (Basingstoke, UK). They are listed in Table 2. All tests were carried out on Muller Hinton agar (Oxoid, Basingstoke, UK) according to the Clinical and Laboratory Standards Institute (CLSI) standards (CSLI, 2013). As there are no CLSI susceptibility breakpoints available for *R. pickettii* or *R. insidiosa,* the antibiotic susceptibility results were interpreted using the CLSI criteria for *Pseudomonas* sp., *Burkholderia cepacia* (for tetracycline) and *Acinetobacter* spp. were used. Zone diameters for the disc susceptibility tests were measured with a Venier Callipers. All testing was carried out in triplicate and all discs were within their used by date. *Pseudomonas aeruginosa* ATCC27853 was included with each testing session. All results were found to be within recommended limits, demonstrating the validity of the testing procedures used.

MIC determination using Etests

The MICs (Minimum Inhibitory Concentrations) of the sixty-eight isolates of *R. pickettii* and *R. insidiosa* to the twelve antibiotics listed in Table 3 were determined using E-tests (Biodisk, Solna, Sweden) on Muller Hinton (MH) agar. The MH plates were inoculated using a sterile cotton swab with suspensions of the cultures (made to 0.5 McFarlane standard) diluted with sterile Tryptone Soya Broth (Oxoid, Basingstoke, UK) and resulted in confluent growth. The MICs were read after incubation overnight at 35°C. The MIC for the bactericidal drugs (Aztreonam, Ceftazidime, Ciprofloxacin, Cefotaxime, Gentamicin, Meropenem, Ofloxacin, Piperacillin) was defined as the point of intersection between the ellipse edge and the E-test strip where there was complete inhibition of all growth. The MIC for the bacteriostatic drugs (tetracycline, minocycline and sulfamethoxazole/trimethoprim) was read at 80% inhibition. There are no CLSI susceptibility breakpoints for *R. pickettii* or *R. insidiosa,* the antibiotic susceptibility testing results were interpreted

using the CLSI criteria for non-enterobacteriaceae (CLSI, 2013). *Pseudomonas aeruginosa* ATCC27853 was used as a control.

Statistical analysis

Simple linear regression analysis was applied to define linear functions correlating the zone of inhibition (mm) with MICs obtained by the disc diffusion and Etest or agar dilution methods (mg/L). The strength of the linear association between pairs of variables was determined by coefficients of determination (*R*-square): *R*-square ≥ 50%, strong correlation; *R*-square 25–49%, moderate correlation; and *R*-square < 25%, weak correlation. The disk diffusion method was accepted as the reference method. Categorical agreement was defined if the tests results were within the same susceptibility category, and errors of disc diffusion and E-test methods were determined as follows: Very major error; (resistant by disk diffusion method, susceptible by Etest method); major error; (susceptible by disk diffusion method, resistant by Etest method); and minor error; (intermediate result was obtained by one method but not the other) (Tatman-Otkun *et al*., 2005). Percentage errors were calculated based on the total number of isolates which were tested. A good agreement was defined as complete category agreement over 90% and the total of very major and major errors below 5%.

Results

Antimicrobial susceptibility disc diffusion tests

The antibiotyping results of *R. pickettii* and *R. insidiosa* from this study (Table 2) generally agree with the results of a previous reports of antibiotic susceptibility carried on *R. pickettii* and on the antibiotic susceptibility of two isolates of *R. insidiosa*, that infected two immunocompromised individuals in Belgium (Van Der

Beek *et al.,* 2005). Almost all *R. pickettii* isolates were susceptible to ceftazidime (one isolate resistant), ciprofloxacin, cefotaxime (one isolate resistant), ofloxacin, meropenem (five isolates resistant), minocycline (one isolate resistant), sulphamethoxazole/trimethoprim and tetracycline (one isolate resistant). All isolates were resistant to aztreonam, with forty-seven isolates resistant to gentamicin and thirteen isolates resistant to ticarcillin/clavulanic acid mix. Susceptibility profiles for *R. insidiosa* were similar except in relation to tetracycline where fourteen isolates were shown to be resistant. Susceptibility profiles for *Ralstonia* species reported in the literature demonstrate susceptibility to cefotaxime, ciprofloxacin, ofloxacin and trimethoprim/sulfamethoxazole and resistance to gentamicin and aztreonam. The major variation was with relation to tetracycline and where *R. insidiosa* was shown to be susceptible in all cases whereas our results show resistance. Little variation was found due to the environmental niche.

MIC determination using E-tests

The E-test MIC results are presented in the Table 3 and used the CLSI breakpoints for non-enterobacteriaceae for interpretation (CLSI, 2013). Nearly all the isolates of both bacteria were highly resistant to the aminoglycoside gentamicin (>256 μg ml^{-1}, fifty-one isolates of *R. pickettii* and fifteen isolates of *R. insidiosa*) and the ß-lactam antibiotic aztreonam (>256 μg ml^{-1}, fifty-two isolates of *R. pickettii* and fifteen isolates of *R. insidiosa*) and variably resistant to the ticarcillin-clavulanic acid mix (>256 μg ml^{-1}, thirty-one isolates of *R. pickettii* and fourteen isolates of *R. insidiosa*). For the carbapenem meropenem six isolates of *R. pickettii* and one isolate of *R. insidiosa* were found to be resistant and the rest susceptible. All isolates were susceptible to the quinolones (ciprofloxacin and ofloxacin), the tetracyclines (tetracycline and minocycline), the cephalosporins (cefotaxime and ceftazidime), the

folate pathway inhibitor (trimethoprim/sulfamethoxazole) and the extended spectrum beta-lactam antibiotic of the ureidopenicillin class (piperacillin).

Discussion

R. pickettii and *R. insidiosa* are a growing problem in hospital (and industrial) settings due their ability to survive and thrive in water. In our results (Table 4) cefotaxime ($p < 0.001$), ciprofloxacin ($p < 0.001$), ofloxacin ($p = 0.421$), tetracycline ($p = 0.934$) and trimethoprim/sulfamethoxazole ($p < 0.001$) showed 100% correlation of the disk diffusion and MIC results for *R. pickettii*. Aztreonam ($p < 0.001$), ciprofloxacin ($p < 0.05$), meropenem ($p < 0.001$), minocycline ($p = 0.818$), ofloxacin ($p = 0.069$) and trimethoprim/sulfamethoxazole ($p < 0.05$) showed 100% correlation of the disk diffusion and MIC results for *R. insidiosa.* Poor correlation rates were found with ceftazidime, meropenem, piperacillin and ticarcillin/clavulanic acid for *R. pickettii* and tetracycline for *R. insidiosa.*

Major differences between the piperacillin, cefotaxime and minocycline results however some major differences were found for ticarcillin/clavulanic acid with 55% of isolates presenting resistance with the E-test and only 25% of isolates being resistant by the disk diffusion method. Major differences were also found with the results for tetracycline where 86% of *R. insidiosa* isolates were found to be resistant by the disk diffusion method, whereas only 13.3% of *R. insidiosa* isolates were found to be resistant by E-test method. Other minor differences were found with the meropenem, gentamicin and ceftazidime results. The results were the same for trimethoprim/sulfamethoxazole, ciprofloxacin, ofloxacin and aztreonam. Similar variation between MIC and disk diffusion results was found in a comparable study that was carried out on *Stenotrophomonas maltophilia* (Nicodemo *et al.,* 2004).

No major differences were observed between the MIC's of different *R. pickettii* isolates from differing environmental niches except for the ticarcillin-clavulanic acid mix where the isolates from the industrial purified water supplies and the purchased isolates were all resistant whereas the clinical isolates, hospital and laboratory purified water isolates were susceptible. This is interesting as it suggests the possibility of a mechanism of resistance in environmental isolates that is not present in clinical isolates.

The MICs of some antibiotics found in the present study were similar to those reported previously for *R. pickettii*. For example Sader and Jones, Gales *et al*., and Fung-Tomc *et al*., reported the MIC_{50} and the MIC_{90} of ceftazidime as > 16 μg ml^{-1} whereas our isolates had an MIC_{50} of 4 μg ml^{-1} and an MIC_{90} of 12 μg ml^{-1}. This was also true for ciprofloxacin, meropenem, trimethoprim/sulfamethoxazole and tetracycline, where similar levels of resistance were found to those in our study (Fung-Tomc *et al.,* 1997; Sader and Jones 2005; Gales *et al*., 2005). These studies were however carried out with a limited number of clinical isolates (thirty-eight, ten and fourteen respectively) compared with our study's fifty-three environmental and clinical isolates.

Resistance to the ticarcillin-clavulanic acid mix in some strains could possibly be due to the presence of the bla_{OXA-22} and bla_{OXA-60} genes which have been identified in both *R. pickettii* 12J (Rpic_3817 and Rpic_3962) and 12D (Rpic12D_3930 and Rpic12D_4075) genomes. Mutational studies have shown that these genes confer resistance to ticarcillin and to a ticarcillin-clavulanic acid mix. These bla_{OXA-22} and bla_{OXA-60} gene products have also been shown to raise MIC values for carbapenems such as meropenem and imipenem and for cephalosporins such as cefepime and cefotaxime (Nordmann *et al.,* 2000; Girlich *et al.,* 2004).

Resistance to aztreonam in gram negative bacteria (like *R. pickettii* and *R. insidiosa*) is usually due to extended-spectrum β-lactamases (Franceschini *et al.,* 1998). Several genes for these extended-spectrum β-lactamases can be found in the genomes *of R. pickettii* 12J (21 proteins) and 12D (20 proteins).

Resistance to the aminoglycosides (like gentamicin) could possibly be due to a multidrug efflux pump found in both the genomes of *R. pickettii* 12J (Rpic_3744-Rpic_3747) and 12D (Rpic12D_3421-Rpic12D_3424) that is similar to that of the BpeAB-OprB (bpeR 45% similarity, bpeA 53% similarity, bpeB 58% similarity and OprB 52% similarity) and AmrAB-OprA (AmrR 36% similarity, AmrA 48% similarity, AmrB 55% similarity and OprA 47% similarity) pumps of *Burkholderia pseudomallei* (Chan *et al.,* 2004; Mima *et al.,* 2010). In some strains of *B. pseudomallei* the BpeAB-OprB system was found to mediate aminoglycoside resistance, while in others the AmrAB-OprA was found to carry out the same task.

These results indicate that sulfamethoxazole/trimethoprim and the fluoroquinolone ciprofloxacin are the best antibiotics with which to treat infections with these bacteria. This agreed with the literature data, which showed that ciprofloxacin worked to treat *R. pickettii* infections after the failure of other treatments (Kendirli *et al.,* 2004; Woo *et al.,* 2002). However issues such as pharmacokinetics and pharmacodynamics and clinical experience should be taken into consideration when choosing the best antibiotic to treat infections.

This is the first in-depth study on the antibiotic resistance of a significant number of *R. pickettii* isolates, which is vital in determining the appropriate treatment in cases of infection. Our results suggest that infection with *R. pickettii* and/or *R. insidiosa* could be treated orally with quinolones or trimethoprim/sulfamethoxazole, which would reduce the invasiveness of treatment (Table 3). In depth clinical studies are essential

to confirm the *in vivo* effectiveness of these antibiotics for the treatment of *R. pickettii*/*R. insidiosa* infections, and to assess the correlation between the susceptibility testing results and the clinical outcomes of treatment. These results indicate that there is little difference in antibiotic resistance between clinical and environmental isolates of the two bacteria; this is of interest as most clinical infections with *R. pickettii* are thought to come from environmental sources such as purified water supplies.

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References:

Adley, C.C., & F.M Saieb (2005). Biofilm formation in high purity water: *Ralstonia pickettii* a special case for analysis. *Ultrapure Water* **22**, 14-18.

Adley, C.C., M.P. Ryan, J.T. Pembroke & F.M. Saieb (2005). *Ralstonia pickettii* in high purity water. *Biofilms: Persistence and Ubiquity*. Mc Bain, A., J. Pratten, D. Spratt, M. Upton, & J. Verran. Cardiff, Biofilm Club**:** 261-272.

Anderson, R. L., B.W. Holland, J. K. Carr, W.W. Bond & M.S. Favero (1990). Effect of disinfectants on pseudomonads colonized on the interior surface of PVC pipes. *Am J Public Health* **80**(1), 17-21.

Bohus, V., E. M. Toth, A. J. Szekely, J. Makk, K. Baranyi, G. Patek, J. Schunk & K. Marialigeti (2010). Microbiological investigation of an industrial ultra-pure supply water plant using cultivation-based and cultivation-independent methods. *Water Research* **44**(20), 6124-6132.

Chan, Y.Y., T.M. Tan, Y.M. Ong & K. L. Chua (2004). BpeAB-OprB, a multidrug efflux pump in *Burkholderia pseudomallei*. *Antimicrob Agents Chemother* **48**(4), 1128-1135.

Clinical and Laboratory Standards Institute CLSI (2013). Performance standards for antimicrobial susceptibility testing; Eighteenth Informational Supplement; CLSI document M100-S23. , Wayne, PA, USA.

Coenye, T., J. Goris, P. De Vos, P. Vandamme & J.J. LiPuma (2003). Classification of *Ralstonia pickettii*-like isolates from the environment and clinical samples as *Ralstonia insidiosa* sp. nov. *Int J Syst Evol Microbiol* **53**(Pt 4), 1075-1080.

Falcone-Dias, M.F., I. Vaz-Moreira & C.M. Manaia (2012). Bottled mineral water as a potential source of antibiotic resistant bacteria. *Water Research* **46**(11), 3612-3622.

Franceschini, N., M. Perilli, B. Segatore, D. Setacci, G. Amicosante, A. Mazzariol & G. Cornaglia (1998). Ceftazidime and aztreonam resistance in *Providencia stuartii*: characterization of a natural TEM-derived extended-spectrum beta-lactamase, TEM-60. *Antimicrob Agents Chemother* **42**(6), 1459-1462.

Fung-Tomc, J., K. Bush, B. Minassian, B. Kolek, R. Flamm, E. Gradelski & D. Bonner (1997). Antibacterial activity of BMS-180680, a new catechol-containing monobactam. *Antimicrob Agents Chemother* **41**(5), 1010-1016.

Gales, A.C., R. N. Jones, S.S. Andrade & H.S. Sader (2005). Antimicrobial susceptibility patterns of unusual nonfermentative gram-negative bacilli isolated from Latin America: report from the SENTRY Antimicrobial Surveillance Program (1997-2002). *Mem Inst Oswaldo Cruz* **100**(6), 571-577.

Gardner, S. & S.T. Shulman (1984). A nosocomial common source outbreak caused by *Pseudomonas pickettii*. *Pediatr Infect Dis* **3**(5), 420-422.

Girlich, D., T. Naas & P. Nordmann (2004). OXA-60, a chromosomal, inducible, and imipenem-hydrolyzing class D beta-lactamase from *Ralstonia pickettii*. *Antimicrob Agents Chemother* **48**(11), 4217-4225.

Heagney MA (1998). An unusual case of bacterial meningitis caused by *Burkholderia pickettii. Clin Micro Newsl* **20,** 102-3.

Han, X.Y. & R.A. Andrade (2005). *Brevundimonas diminuta* infections and its resistance to fluoroquinolones. *J Antimicrob Chemother* **55**(6), 853-859.

Hoefel, D., P. T. Monis, W. L. Grooby, S. Andrews & C. P. Saint (2005). Profiling bacterial survival through a water treatment process and subsequent distribution system. *J Appl Microbiol* **99**(1), 175-186.

Kendirli, T., E. Ciftci, E. Ince, S. Incesoy, H. Guriz, A. D. Aysev, E. Tutar, G. Yavuz & U. Dogru (2004). *Ralstonia pickettii* outbreak associated with contaminated distilled water used for respiratory care in a paediatric intensive care unit. *J Hosp Infect* **56**(1), 77-78.

Koenig, D.W. & D.L. Pierson (1997). Microbiology of the space shuttle water system. *Water Science and Technology* **35**(11-12), 59-64.

Kulakov, L.A., M. B. McAlister, K. L. Ogden, M. J. Larkin & J. F. O'Hanlon (2002). Analysis of bacteria contaminating ultrapure water in industrial systems. *Applied and Environmental Microbiology* **68**(4), 1548-1555.

Labarca, J.A., W.E. Trick, C.L. Peterson, L.A. Carson, S.C. Holt, M.J. Arduino, M. Meylan, L. Mascola & W.R. Jarvis (1999). A multistate nosocomial outbreak of *Ralstonia pickettii* colonization associated with an intrinsically contaminated respiratory care solution. *Clin Infect Dis* **29**(5), 1281-1286.

Lee, J., C. S. Lee, K. M. Hugunin, C.J. Maute & R.C. Dysko (2010). Bacteria from drinking water supply and their fate in gastrointestinal tracts of germ-free mice: A phylogenetic comparison study. *Water Research* **44**(17), 5050-5058.

Maki, D.G., B. S. Klein, R. D. McCormick, C.J. Alvarado, M.A. Zilz, S. M. Stolz, C.A. Hassemer, J. Gould & A.R. Liegel (1991). Nosocomial *Pseudomonas pickettii* bacteremias traced to narcotic tampering. A case for selective drug screening of health care personnel. *JAMA* **265**(8), 981-986.

McAlister, M.B., L.A. Kulakov, J.F. O'Hanlon, M.J. Larkin & K.L. Ogden (2002). Survival and nutritional requirements of three bacteria isolated from ultrapure water. *J Ind Microbiol Biotechnol* **29**(2), 75-82.

McNeil, M.M., S.L. Solomon, R.L. Anderson, B.J. Davis, R.F. Spengler, B.E. Reisberg, C. Thornsberry & W.J. Martone (1985). Nosocomial *Pseudomonas pickettii* colonization associated with a contaminated respiratory therapy solution in a special care nursery. *J Clin Microbiol* **22**(6), 903-907.

Mima, T. & H.P. Schweizer (2010). The BpeAB-OprB efflux pump of *Burkholderia pseudomallei* 1026b does not play a role in quorum sensing, virulence factor production, or extrusion of aminoglycosides but is a broad-spectrum drug efflux system. *Antimicrob Agents Chemother* **54**(8), 3113-3120.

Nicodemo, A.C., M.R. Araujo, A.S. Ruiz & A.C. Gales (2004). In vitro susceptibility of *Stenotrophomonas maltophilia* isolates: comparison of disc

diffusion, E-test and agar dilution methods. *J Antimicrob Chemother* **53**(4), 604-608.

Nordmann, P., L. Poirel, M. Kubina, A. Casetta & T. Naas (2000). Biochemical-genetic characterization and distribution of OXA-22, a chromosomal and inducible class D beta-lactamase from *Ralstonia* (*Pseudomonas*) *pickettii*. *Antimicrob Agents Chemother* **44**(8), 2201-2204.

Orlíková, H., J. Prattingerová, H. Žemličková, V. Melicherčíková, J. Urban & M. Sochorová (2011). [Bacteremia and sepsis caused by *Ralstonia insidiosa* (*Ralstonia pickettii*-like) in dialysis patients in a Czech hospital in the period January-May 2011].*Zprávy Centra epidemiologie a mikrobiologie* **20**(8), 290-294.

Penna, V.T., S.A. Martins & P.G. Mazzola (2002). Identification of bacteria in drinking and purified water during the monitoring of a typical water purification system. *BMC Public Health* **2**, 13.

Raveh, D., A. Simhon, Z. Gimmon, T. Sacks & M. Shapiro (1993). Infections caused by *Pseudomonas pickettii* in association with permanent indwelling intravenous devices: four cases and a review. *Clin Infect Dis* **17**(5), 877-880.

Roberts, L.A., P.J. Collignon, V.B. Cramp, S. Alexander, A.E. McFarlane, E. Graham, A. Fuller, V. Sinickas & A. Hellyar (1990). An Australia-wide epidemic of *Pseudomonas pickettii* bacteraemia due to contaminated "sterile" water for injection. *Med J Aust* **152**(12), 652-655.

Ryan, M.P., J.T. Pembroke & C.C. Adley (2006). *Ralstonia pickettii*: a persistent gram-negative nosocomial infectious organism. *J Hosp Infect* **62**(3), 278-284.

Ryan, M.P., J.T. Pembroke & C.C. Adley (2007). *Ralstonia pickettii* in environmental biotechnology: potential and applications. *J Appl Microbiol* **103**(4), 754-764.

Ryan, M.P., J.T. Pembroke & C.C. Adley (2009). Novel Tn*4371*-ICE like element in *Ralstonia pickettii* and genome mining for comparative elements. *BMC Microbiol* **9**, 242.

Ryan, M.P., J.T. Pembroke & C.C. Adley (2011a). Differentiating the growing nosocomial infectious threats *Ralstonia pickettii* and *Ralstonia insidiosa*. *Eur J Clin Microbiol Infect Dis* **30**(10), 1245-1247.

Ryan, M.P., J.T. Pembroke & C.C. Adley (2011b). Genotypic and phenotypic diversity of *Ralstonia pickettii* and *Ralstonia insidiosa* isolates from clinical

and environmental sources including High-purity Water. Diversity in *Ralstonia pickettii*. *BMC Microbiol* **11**, 194.

Ryan, M.P., Pembroke, J.T. and Adley, C.C. (2011c). *Ralstonia* (Chapter 66). *Molecular Detection of Human Bacterial Pathogens*. D. Liu, Francis & Taylor CRC Press.

Sader, H.S. & R.N. Jones (2005). Antimicrobial susceptibility of uncommonly isolated non-enteric Gram-negative bacilli. *Int J Antimicrob Agents* **25**(2), 95-109.

Strateva, T., Kostyanev, T. & Setchanova, L. (2012). *Ralstonia pickettii* sepsis in a hemodialysis patient from Bulgaria. *Braz J Infect Dis* **16,** 400-401.

Sundaram, S., M. Auriemma, G. Howard, Jr., H. Brandwein & F. Leo (1999). Application of membrane filtration for removal of diminutive bioburden organisms in pharmaceutical products and processes. *PDA J Pharm Sci Technol* **53**(4), 186-201.

Sundaram, S., M. Lewis, J. Eisenhuth, G. Howard, Jr. & B. Larson (2002). Method for qualifying microbial removal performance of 0.1 micron rated filters. Part IV: Retention of *Hydrogenophaga pseudoflava* (ATCC 700892) and *Ralstonia pickettii* (ATCC 700591) by 0.2 and 0.22 micron rated filters. *PDA J Pharm Sci Technol* **56**(3), 150-171.

Szymanska, J. (2006). Bacterial decontamination of DUWL biofilm using Oxygenal 6. *Ann Agric Environ Med* **13**(1), 163-167.

Tatman-Otkun, M., S. Gurcan, B. Ozer, B. Aydoslu & S. Bukavaz (2005). The antimicrobial susceptibility of *Stenotrophomonas maltophilia* isolates using three different methods and their genetic relatedness. *BMC Microbiology* **5**:24.

Van der Beek, D., K. Magerman, G. Bries, A. Mewis, P. Declercq, V. Peeters, J. L. Rummens, M. Raymaekers & R. Cartuyvels (2005). Infection with *Ralstonia insidiosa* in two patients. *Clinical Microbiology Newsletter* **27**(20), 159-161.

Van Houdt, R., Toussaint, A., Ryan, M.P., J.T. Pembroke, M. Mergeay & C.C. Adley (2012). The Tn*4371* ICE Family of Bacterial Mobile Genetic Elements. *Bacterial Integrative Mobile Genetic Elements*. A. P. Roberts & P. Mullany. Austin (TX), Landes Bioscience.

Wertheim, W. A. & Markovitz, D. M. (1992). Osteomyelitis and Intervertebral Discitis Caused by Pseudomonas-Pickettii. *J Clin Microbiol* **30,** 2506-2508.

Woo, P.C., S.S. Wong & K. Y. Yuen (2002). *Ralstonia pickettii* bacteraemia in a cord blood transplant recipient. *New Microbiol* **25**(1), 97-102.

Zellweger, C., T. Bodmer, M.G. Tauber & K. Muhlemann (2004). Failure of ceftriaxone in an intravenous drug user with invasive infection due to *Ralstonia pickettii*. *Infection* **32**(4), 246-248.

Table 1: *Ralstonia* Strains used in this work

Strain	Source
R. pickettii JCM5969, NCTC11149, DSM6297, CIP73.23 CCUG3318	Culture Collections
R. pickettii CCM2846, CCUG18841	Culture Collections
R. insidiosa ATCC4199	Culture Collection
R. insidiosa LMG21421	Culture Collection
R. pickettii ULC193, ULC194, ULC277, ULC297, ULC298, ULC224, ULC421	Isolated from the lung sputum of Cystic Fibrosis Patients at the Microbiology laboratory of Limerick Regional Hospital, Limerick Ireland (2002-2003)
R. pickettii ULCSF1	Isolated from Cerebrospinal fluid at the University of Pittsburgh Medical Center, Pittsburgh, USA (2009)
R. pickettii ULI785, ULI788, ULI790, ULI791, ULI796, ULI800, ULI801, ULI804, ULI806, ULI807, ULI818, ULI159, ULI162, ULI165, ULI167, ULI169, ULI171, ULI174, ULI181, ULI187, ULI188, ULI193	Isolated from various Industrial Purified water systems (Ireland)
R. insidiosa ULI821, ULI797, ULI785, ULI181, ULI794, ULI185, ULI166, ULI819, ULI784, ULI163, ULI795	Isolated from various Industrial Purified water systems (Ireland)
R. pickettii ULM001, ULM002, ULM003, ULM004, ULM005, ULM006	Isolated from various Millipore Purified water systems (France)
R. pickettii ULM007, ULM008, ULM009, ULM010, ULM011	Isolated from various Millipore Laboratory Purified water systems (Ireland)
R. insidiosa ULM008, ULM009	Isolated from various Millipore Laboratory Purified water systems (Ireland)
R. pickettii 12J, 12D	Heavy Metal Polluted lake, USA
R. pickettii 1177203, 1179198, 1179199, 1179204, 1179250, 1179251	Endoscopy unit washing water, UK

Table 2: Antibiotic resistance profile of 53 *Ralstonia pickettii* and 15 *Ralstonia insidiosa* isolates using disk diffusion testing

	Atm	**Caz**	**Cip**	**Cn**	**Ctx**	**Men**	**Mh**	**Ofl**	**Prl**	**SxT**	**Te**	**Tic**
R. pickettii (n=53)	53 (100%)	5 (9%)	-	48 (91%)	-	7 (13%)	1 (2%)	-	-	-	-	30 (57%)
R. insidiosa (n=15)	15 (100%)	-	-	14 (93%)	-	2 (13%)	-	-	-	-	13 (86%)	12 (80%)

Atm-Aztreonam 30 µg ml^{-1}; Caz-Ceftazidime 30 µg ml^{-1}; Cip-Ciprofloxacin 5 µg ml^{-1}; Ctx-Cefotaxime 30 µg ml^{-1}; Cn-Gentamicin 10 µg ml^{-1}; Men-Meropenem 10 µg ml^{-1}; Mh-Minocycline 30 µg ml^{-1}; Te-Tetracycline 30 µg ml^{-1}; Ofl-Ofloxacin 5 µg ml^{-1}; Prl-Piperacillin 100 µg ml^{-1}; SxT-Sulphamethoxazole/trimethoprim 23.75/1.25 µg ml^{-1}; Tic-Ticarcillin/clavulanic acid 75/10 µg ml^{-1}

CLSI Breakpoints for resistance for *Pseudomonas aeruginosa* (Table 2B-1) antibiotic except for ceftazidime, meropenem, minocycline and Trimethoprim/sulfamethoxazole where Breakpoints for resistance for *Burkholderia cepacia* (Table 2B-3) and cefotaxime and tetracycline where Breakpoints for resistance for *Acinetobacter* spp. (Table 2B-2) were used (CSLI, 2013)

12 **Table 3:** $MIC_{50,}$ MIC_{90} and ranges for 53 *Ralstonia pickettii* and 15 *Ralstonia insidiosa* isolates (All values in $\mu g\ ml^{-1}$)

		Ralstonia pickettii (*n*=53)				*Ralstonia insidiosa* (*n*=15)			
Antibiotic	**Breakpoint for resistance ***	**MIC_{50} #**	**MIC_{90} #**	**Range**	**Percentage resistant**	**MIC_{50} #**	**MIC_{90} #**	**Range**	**Percentage resistant**
Aztreonam	≥32	>256	>256	24->256	99.9	>256	>256	>256	100
Cefotaxime	≥64	0.5	1	0.03-2	0	1	1	0.25-1	0
Ceftazidime	≥32	4	12	0.75-21	9.4	4	6	3-8	0
Ciprofloxacin	≥4	0.06	0.12	0.002-0.12	0	0.12	0.12	0.06-0.12	0
Gentamicin	≥16	>256	>256	0.06->256	99.9	>256	>256	>256	100
Meropenem	≥16	2	8	0.125->256	13.2	2	4	0.032->256	13.3
Minocycline	≥16	0.38	1.0	0.16-1.0	0	1	1	0.19-1	0
Ofloxacin	≥8	0.25	0.5	0.006-6	0	0.38	0.5	0.25-0.5	0
Piperacillin	≥128	3	16	0.092-18	2	8	12	0.064-16	0
Tetracycline	≥16	1.5	3	0.023-3	0	3	3	0.38-6	13.3
Ticarcillin/clavulanic acid	≥128/2	>256	>256	0.016->256	55	>256	>256	>256	93.3
Trimethoprim/sulfamethoxazole	≥4/76	0.012	0.023	0.002-0.38	0	0.012	0.023	0.008-0.032	0

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14 * CLSI Breakpoints for resistance for non-enterobacteriaceae (Table 2B-5) were used (CSLI, 2013)

15 # MIC_{50} and MIC_{90} defined as the minimal concentration of antibiotic capable of inhibiting 50% and 90% of the isolates tested, respectively

16 **Table 4:** Categorical agreement and error rates for strains of *Ralstonia pickettii* and *Ralstonia insidiosa* tested

	Ralstonia pickettii (*n*=53)				*Ralstonia insidiosa* (*n*=15)			
Antibiotic	**Very Major%**	**Major%**	**Minor%**	**% Correlation**	**Very Major%**	**Major%**	**Minor%**	**% Correlation**
Aztreonam	0	0	1.88	98.12	0	0	0	100
Cefotaxime	0	0	0	100	6.67	0	0	93.33
Ceftazidime	9.43	0	39.62	50.95	0	0	6.67	93.33
Ciprofloxacin	0	0	0	100	0	0	0	100
Gentamicin	0	5.66	0	94.34	0	6.67	0	93.33
Meropenem	11.32	9.43	20.75	58.5	0	0	0	100
Minocycline	0	0	7.55	92.45	0	0	0	100
Ofloxacin	0	0	0	100	0	0	0	100
Piperacillin	0	0	30.19	69.81	0	0	60	40
Tetracycline	0	0	0	100	86.67	0	0	13.33
Ticarcillin/clavulanic acid	16.98	0	47.17	35.85	0	0	20	80
Trimethoprim/sulfamethoxazole	0	0	0	100	0	0	0	100

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