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Presence of *Stenotrophomonas maltophilia* exhibiting high genetic similarity to clinical isolates in final effluents of pig farm wastewater treatment plants

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ABSTRACT

Although the prevalence of community-acquired *Stenotrophomonas maltophilia* infections is sharply increasing, the sources and likely transmission routes of this bacterium are poorly understood. We studied the significance of the presence of *S. maltophilia* in final effluents and receiving rivers of pig farm wastewater treatment plants (WWTPs). The loads and antibiotic resistance profiles of *S. maltophilia* in final effluents were assessed. Antibiotic resistance determinants and biofilm formation genes were detected by PCR, and genetic similarity to clinical isolates was investigated using multilocus sequence typing (MLST). *S. maltophilia* was recovered from final effluents at two of three farms and one corresponding receiving river. Tests of resistance to antibiotics recommended for *S. maltophilia* infection revealed that for each agent, at least one isolate was classified as resistant or intermediate, with the exception of minocycline. Furthermore, multidrug resistant *S. maltophilia* susceptible to antibiotics of only two categories was isolated and found to carry the *sul2* gene, conferring trimethoprim/sulfamethoxazole resistance. All isolates carried *spgM*, encoding a major factor in biofilm formation. MLST revealed that isolates of the same sequence type (ST; ST189) were present in both effluent and receiving river samples, and phylogenetic analysis showed that all of the STs identified in this study clustered with clinical isolates. Moreover, one isolate (ST192) recovered in this investigation demonstrated 99.61% sequence identity with a clinical isolate (ST98) associated with a fatal infection in South Korea. Thus, the pathogenicity of the isolates reported here is likely similar to that of those from clinical environments, and WWTPs may play a role as a source of *S. maltophilia* from which this bacterium spreads to human communities. To the best of our knowledge, this represents the first report of *S. maltophilia* in pig farm WWTPs. Our results indicate that nationwide epidemiological investigations are needed to examine the possible link between WWTP-derived *S. maltophilia* and hospital- and community-acquired infections.

1. Introduction

Wastewater treatment plants (WWTPs) are widely regarded as “hotspots” for the development of antibiotic resistance among bacteria (Rizzo et al., 2013). At these sites, antibiotic resistance can be acquired by the transfer of resistance determinants from donor to recipient bacteria or may result from selective pressure exerted by the residual presence of antibiotics (Li et al., 2010; Yan et al., 2017; Yang et al., 2016). If not completely eradicated, resistant bacteria will then be discharged into the wider terrestrial and aquatic environment. *Stenotrophomonas maltophilia* is known to be able to survive in activated sludge, the preferred treatment method in WWTPs (Ivanov et al., 2005). Moreover, this bacterium can resist exposure to a series of additional treatment processes, such as chlorine or UV disinfection, by export of toxic metabolites from the periplasm through efflux pumps or the formation of a biofilm, which acts as a barrier against adverse conditions (Brooke, 2012; Di Bonaventura et al., 2010; Pompilio et al., 2008). Therefore, *S. maltophilia* is likely to not only survive WWTP processes, but also be discharged in final effluent into the aquatic environment carrying various antibiotic resistance determinants, ultimately reaching humans.

S. maltophilia, previously known as *Pseudomonas maltophilia* and later *Xanthomonas maltophilia*, is an emerging opportunistic pathogen that typically infects the respiratory tract, particularly in patients with cystic fibrosis and immunocompromised individuals (Brooke, 2012; Denton and Kerr, 1998). This bacterium exhibits intrinsic and/or

Abbreviations: WWTPs, wastewater treatment plants; NGB, non-enteric gram-negative bacterium; TSB, tryptic soy broth; TSA, tryptic soy agar; MLST, multilocus sequence typing; ST, sequence type; MDR, multidrug resistant

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acquired resistance to commonly used antimicrobial agents such as penicillin, cephalosporin, monobactams, aminoglycosides, and even carbapenem and colistin, which are regarded as the last resort for treating serious bacterial infections, considerably limiting treatment options for *S. maltophilia* infections (Chang et al., 2004; Liaw et al., 2010; Walsh et al., 2011). This considerable drug resistance derives from a variety of determinants, including resistance-nodulation-division efflux pumps, multiple β-lactamases, and biofilm formation ability (Brooke, 2012; Nicodemo and Paez, 2007). The most effective antibiotic against *S. maltophilia* is trimethoprim/sulfamethoxazole, being associated with the lowest resistance rate (~5%), followed by fluoroquinolones (6.5–14.1%), and polymyxins (20–32%) (Sader and Jones, 2005). Trimethoprim/sulfamethoxazole therefore remains the therapy of choice for *S. maltophilia* infections worldwide. However, recent studies have described isolates resistant to this antimicrobial due to acquisition of the *sul1* or *sul2* gene as part of the class 1 integron, leaving no treatment choices (Barbolla et al., 2004; Chung et al., 2015; Hu et al., 2011; Toleman et al., 2007).

According to recent international surveys of antimicrobial resistance among bacteria, such as the SENTRY surveillance program (JMI Laboratories, 2017; https://www.jmilabs.com/sentry-surveillance-program/) and Study for Monitoring Antimicrobial Resistance Trends (International Federation of Pharmaceutical Manufacturers and Associations, 2017; http://partnerships.ifpma.org/partnership/study-for-monitoring-antimicrobial-resistance-trends-smart), *S. maltophilia* is the third most prevalent non-enteric gram-negative bacterium (NGB) associated with hospital-acquired infections (accounting for 59.2% of all such NGB infections), behind *Acinetobacter* spp. and *Pseudomonas aeruginosa* (Chang et al., 2015; Sader and Jones, 2005). Although this bacterium is generally regarded as a nosocomial pathogen, the incidence of community-acquired *S. maltophilia* infections is also drastically increasing. For instance, it has recently been reported to be responsible for 17.6 and 23.7% of all community-acquired bloodstream infections in Taiwan and France, respectively (Chang et al., 2015). Therefore, extensive epidemiological studies should be conducted to establish likely routes of *S. maltophilia* transmission, in both hospital and environmental settings. However, to the best of our knowledge, there have been no prior reports of the presence and characterization of *S. maltophilia* in the final effluents and corresponding receiving rivers of WWTPs serving pig farms, which use the largest quantities of antibiotics of all animal husbandry facilities worldwide (Barton, 2014; Novais et al., 2013).

The aims of this study were to investigate (1) *S. maltophilia* loads in final effluents and receiving rivers of pig farm WWTPs using activated sludge, (2) resistance of *S. maltophilia* to antibiotics currently used in clinical practice, and (3) the distribution of biofilm formation genes and horizontally transmitted resistance genes. In addition, we investigated the genetic diversity present among the *S. maltophilia* isolates recovered and assessed their genetic similarity to all reported clinical isolates from South Korea and environmental isolates regardless of country of origin.

2. Materials and methods

2.1. Sample collection

The WWTP process and the points at which samples were taken are illustrated in Fig. 1. From October 2016 to February 2017, a total of 45 final effluent samples were collected from three farms in Gyeong-gi Province, South Korea (5 visits separated by 1-month intervals × 3 samples per visit separated by 4-h intervals × 3 farms). Simultaneously, a total of 30 corresponding receiving river samples were collected 100 m upstream and 150–200 m downstream of the junction between the river and the WWTP at each farm (5 visits × 2 sampling points [upstream and downstream] × 3 farms). The pig farm owners were invited in person or by telephone to provide information concerning the number of pigs raised, antibiotic usage, and wastewater treatment type. All of the pig farm WWTPs employed conventional activated sludge, including pre-separation, anaerobic, and aerated treatment, and coagulant sedimentation steps. After treatment, effluents were directly discharged into the receiving rivers at a rate of approximately 30,000–40,000 m^3/day without additional disinfection.

2.2. Isolation and enumeration of bacteria

S. maltophilia was isolated and enumerated as described previously (Brooke, 2012; Denton and Kerr, 1998). Five hundred milliliters of each sample was concentrated by filtration through a sterile 0.45-μm membrane filter (Millipore, Billerica, MA, USA), which was then placed on MacConkey agar (Oxoid, Basingstoke, UK) supplemented with 1 μg/mL imipenem (Sigma, St. Louis, MO, USA), and incubated at 30 °C for 48 h. To avoid confounding effects, we analyzed only one colony per agar plate, selected according to colony morphology and pigmentation (yellowish to greenish). Suspected *S. maltophilia* colonies were counted and recorded simultaneously. When *S. maltophilia* was not detected in the quantitative test, qualitative analysis was conducted using an enrichment culture established by placing the membrane filter in tryptic soy broth (TSB; Oxoid). Colonies were sub-cultured on tryptic soy agar (TSA; Oxoid) and stored at −70 °C before analysis. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All isolates were thereafter identified by 16S rRNA sequencing. In addition, as an indicator of contamination of final effluent with fecal bacteria, *Escherichia coli* was enumerated simultaneously using 0.45-μm membrane filters (Watkinson et al., 2007). The detection limit for both *S. maltophilia* and *E. coli* was 1 CFU/500 mL. Bacteria that grew on the imipenem-containing MacConkey agar but exhibited a colony morphology different from that of *S. maltophilia* were also isolated and identified using 16S rRNA sequencing. Such bacteria were only obtained from enriched final effluent samples, i.e., they were not quantified.

2.3. Antibiotic susceptibility tests

The antibiotic susceptibility of the *S. maltophilia* isolates recovered was evaluated using an AST-N225 card with the VITEK 2 system (bioMérieux, Marcy l'Etoile, France). This card contains the following antibiotics recommended for infections with *S. maltophilia* (group A) and non-Enterobacteriaceae (group B), according to Clinical and Laboratory Standards Institute (CLSI) 2016 guidelines (document M100S): group A: ticarcillin/clavulanic acid, ceftazidime, ciprofloxacin, minocycline, and trimethoprim/sulfamethoxazole; group B: ampicillin/sulbactam, piperacillin, piperacillin/tazobactam, cefotaxime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tigecycline, and colistin. Susceptibility to group A antibiotics was verified using the agar dilution method (Wiegand et al., 2008). Isolates were considered susceptible, intermediate, or resistant following CLSI 2016 guidelines (Clinical and Laboratory Standards Institute, 2016). *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as control strains.

2.4. PCR analysis of acquired antibiotic resistance genes and biofilm formation genes

The *S. maltophilia* isolates obtained were evaluated for the presence of the horizontally transmissible resistance genes *bla*SPM, *bla*IMP, *bla*VIM, *bla*KPC, *bla*NDM, and *bla*OXA-48 (conferring carbapenem resistance), and *mcr-1* (conferring colistin resistance). Carriage of class 1 and class 2 integrons and the biofilm formation genes *spgM*, *rmlA*, and *rpfF* was also tested. The primer pairs and amplification conditions used for PCR in the present study are shown in Table S1.

2.5. Transconjugation assay

Transconjugation was performed for all isolates using the azide-

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