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Spread of multidrug-resistant *Escherichia coli* harboring integron via swine farm waste water treatment plant

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ABSTRACT

Wastewater treatment plants (WWTPs) that release treated wastewater into the environment have emerged as a major threat to public health. In this study, we investigated *Escherichia coli* load and antibiotic-resistance profiles across different treatment processes at a swine farm WWTP. The frequency of the detection of class 1 and 2 integrons, and their association with antibiotic resistance, were also analyzed. Samples were obtained at each of five sampling sites that represented each processing step within the WWTP. The largest decrease in *E. coli* load was observed during the anaerobic digestion step (from 4.86 to 2.89 log CFU/mL). Isolates resistant to β-lactam antibiotics were efficiently removed after a series of treatment steps, whereas the proportions of isolates resistant to non-β-lactam antibiotics and multidrug-resistant strains were maintained across treatments. The occurrence of integron-positive strains was not significantly different at the various sampling sites (43.4–70%; $p > 0.05$). Of the class 1 integron-positive isolates, 17.9% harbored the integron-associated gene cassettes *aadA2*, *aadA12*, *aadA22*, and *dfrA15*. To the best of our knowledge, this is the first description of a class 1 integron containing the *aadA12* gene cassette from a swine farm and the presence of a class 1 integron containing *dfrA15* in *E. coli*. This suggests that novel antibiotic-resistance gene cassette arrays could be generated in swine farm WWTPs. Moreover, 75% of integron-positive strains were categorized as multidrug resistant, whereas only 15.4% of integron-negative strains were multidrug resistant ($p < 0.05$), indicating that integrons may be responsible for mediating resistance in WWTPs. With regard to the occurrence of multidrug-resistant, integron-positive *E. coli* recovered from the final effluent, our results highlighted the potential risks associated with wastewater discharge from swine farm WWTPs in terms of the spread of antibiotic-resistant bacteria to the aquatic environment.

1. Introduction

The World Health Organization (WHO) has identified the emergence and spread of antibiotic resistance in bacteria as a major public health concern. This is primarily due to increased antibiotic resistance in bacteria through the extensive use of antibiotics in animal husbandry (Molton et al., 2013). Swine husbandry in particular is one of the most heavily medicated industries. For example, a previous study performed in the European Union revealed that the worldwide antibiotic consumption in pig production accounts for 60% of all antibiotics used in animals (Mevius et al., 1999). In South Korea from 2011 to 2015, approximately 900 t of antibiotics were sold annually for use in food-producing animals and half of these were used in swine husbandry (KAHPA, 2015). As a consequence, many countries have reported the occurrence of multidrug-resistant bacteria in swine waste (Ewers et al., 2012), suggesting that such material is a serious threat to the environment.

As disseminated antibiotic-resistant bacteria in swine waste ultimately reach wastewater treatment plants (WWTPs), these sites have recently been recognized as “hotspots” that play a critical role in the development and persistence of antibiotic-resistant bacteria (Rizzo et al., 2013). Subsequent selection pressure could also occur because of the presence of residual antibiotics and chemicals (Akiba et al., 2015; Guruge et al., 2015). Horizontal gene transfer by mobile genetic elements, such as plasmids, phages, transposons, and integrons, can give rise to antibiotic resistance in bacteria (Rizzo et al., 2013). If antibiotic-

Abbreviations: AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CZ, cefazolin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; AZT, aztreonam; AK, amikacin; CIP, ciprofloxacin; GN, gentamicin; MDR, multi-drug resistance; ESBL, extended-spectrum β-lactamase; AmpC, AmpC β-lactamase; WHO, World Health Organization; WWTP, wastewater treatment plant; SG, sludge; RI, raw influent; PE, primary effluent; SE, secondary effluent; FE, final effluent

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resistant bacteria are not completely eliminated, it is likely that these organisms will be discarded and spread into both aquatic and terrestrial environments and ultimately enter the human population (Zhang et al., 2015). Moreover, the products of WWTPs, such as biosolids and effluent, are often reused and re-applied for different purposes in agriculture and aquaculture (Lopez et al., 2006). It is therefore important to understand the fates of indicator bacteria and their resistance profiles at each process step in swine farm WWTPs. This includes solid-liquid separation, anaerobic digestion, aerobic digestion, and coagulant sedimentation steps.

Among the various mobile genetic elements found in bacteria, integrons are considered the most important for the spread of antibiotic-resistance in clinical settings and also the environment, including agriculture (Sunde et al., 2015). By capturing and excising antibiotic resistance gene cassettes, integrons are frequently associated with the development of multidrug resistance in gram-negative bacteria. This is particularly true for *Escherichia coli*, especially compared to other indicator bacteria (Sunde, 2005; Sunde et al., 2015). Class 1 integrons are the most common type of integron and consist of two conserved segments flanking the cassette area. The 5′-conserved segment (5′-CS) includes the gene for class 1 integrase (*intI1*) and a recombination site (*attI1*). The 3′-conserved segment (3′-CS) includes the *sul1* gene that confers resistance to sulfamethoxazole. Class 2 integrons are similar to class 1 in that they possess an integrase gene and a recombination site, but lack the *sul1* gene in the 3′-CS. Integrons have been attracting increased research attention recently (Sunde, 2005), although little is known about the role they play in the occurrence of antibiotic-resistant bacteria in swine farm WWTPs.

To address this, this study investigated *E. coli* loads and antibiotic resistance profiles across multiple treatment processes at a swine farm WWTP. The prevalence of class 1 and class 2 integrons, and their inserted gene cassettes, were also assessed to determine any correlation between integron presence and resistance to antibiotics.

2. Materials and methods

2.1. WWTP on a swine farm

For the study, we selected a large commercial swine farm located in Anseong, South Korea. The farm was a farrow-to-finish operation with a sow population of 750. Amoxicillin was the most commonly used antibiotic on the farm used to prevent early mortality due to *E. coli* and *Staphylococcus* spp. infections. The antibiotic was administered continuously in the drinking water or feed based on the manufacturer provided instructions. A summary of the processes at the swine farm WWTP, and sampling points for isolating *E. coli*, are shown in Fig. 1. The WWTP processes were based on an activated sludge method and had multiple treatment steps, including primary treatment (solid-liquid separation), secondary treatment (anaerobic digestion and aerobic digestion), and tertiary treatment (coagulant sedimentation). The hydraulic residence times within the anaerobic digestion and aerobic digestion were 9 and 3 days, respectively. In the WWTP, there was no additional disinfection step for the final effluent. The sludge from the WWTP was sent to a fodder company and treated for reuse as a biosolid in agriculture.

2.2. Sample collection for bacterial analysis

Sampling was performed three times from December 2016 to February 2017. The samples were designated as the solid after solid-liquid separation (sludge; SG), untreated raw waste (raw influent; RI), the liquid sample after solid-liquid separation (primary effluent; PE), the sample after anaerobic digestion (secondary effluent; SE), and the sample after aerobic digestion and coagulant sedimentation (final effluent; FE). On each of the three visits, we collected three samples at each sampling site at 4 h intervals to avoid any confounding effects. SG (50 g) was also collected in 50-mL conical tubes. Samples were transported to the laboratory on ice and processed within 12 h of collection.

2.3. Enumeration and isolation of E. coli

E. coli was enumerated using plate counts and Petri film (3 M Microbiology Products, St. Paul, MN, USA) (BAM, 2013), with some modifications. In the case of the effluent samples, 500 mL of each sample was inoculated onto the Petri film using a membrane filtration method (0.45-μm) to reduce the detection limit, as described previously (Laroche et al., 2009). All samples were appropriately diluted in buffered peptone water and inoculated onto the Petri film. This was followed by incubation at 45.5 °C for 24–48 h (Scheinberg et al., 2017). All blue colonies with gas bubbles (likely to be *E. coli*) were counted and a maximum of three colonies per Petri film were picked from the available colonies for further analysis. The *E. coli* counts were expressed as CFU/mL or g of sample.

2.4. Identification and antibiotic susceptibility tests

The identity and antibiotic susceptibility of the presumptive *E. coli* isolates were determined using a Vitek 2 system (bioMérieux, Marcy l'Etoile, France) with GN and AST-N224 cards, respectively, according to the manufacturer's instructions. The antibiotics used for the susceptibility tests were ampicillin (AMP), amoxicillin/clavulanic acid (AMC), piperacillin/tazobactam (TZP), cefazolin (CZ), cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), aztreonam (AZT), ertapenem (ETP), and imipenem (IMP), amikacin (AK), gentamicin (GN), ciprofloxacin (CIP), tigecycline (TGC), and trimethoprim/sulfamethoxazole (SXT), along with screening for extended-spectrum β-lactamase (ESBL) production. Isolates were considered susceptible, intermediate, or resistant according to guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2008). All ESBL-producing isolates were confirmed using a double-disk synergy test according to guidelines from EUCAST (Christian et al., 2013). Phenotypically, resistance to AMP, AMC, CZ, and FOX among the isolates was considered diagnostic of AmpC β-lactamase (AmpC)-producing bacteria (von Salviati et al., 2015). The criterion for multidrug resistance (MDR) was defined as resistance to at least three different classes of antibiotic, as described previously (Magiorakos et al., 2012).

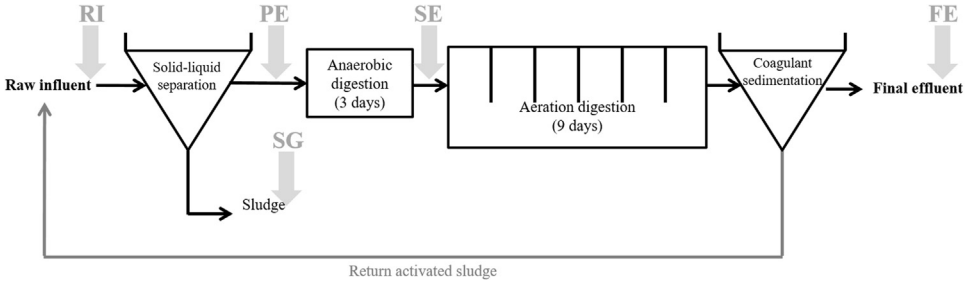

Fig. 1. Schematic showing the WWTP process and the location of the sampling sites. The hydraulic residence times for anaerobic and aerobic digestion were 9 and 3 days, respectively.