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The prevalence of multidrug-resistant *Aeromonas* spp. in the municipal wastewater system and their dissemination in the environment

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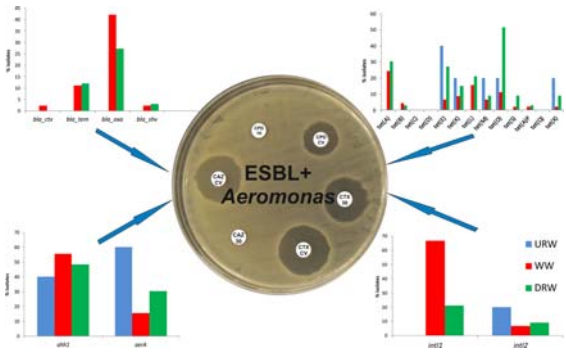
HIGHLIGHTS

- Antibiotic-resistance and virulence determinants of *Aeromonas* spp. were studied.
- Over 72% of analyzed *Aeromonas* isolates were multidrug-resistant.
- Isolates resistant to beta-lactams most frequently harbored bla_{TEM} and bla_{OXA} genes.
- *tet*(E) and *tet*(O) were more frequent genes encoding resistance to tetracyclines.
- Almost 59% of *Aeromonas* spp. harbored at least one virulence gene.

GRAPHICAL ABSTRACT



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ABSTRACT

The objective of this study was to identify the determinants of antibiotic resistance and virulence of *Aeromonas* spp. isolated from treated wastewater (WW) and from samples of river water collected upstream (URW) and downstream (DRW) from the effluent discharge point. The resistance of *Aeromonas* spp. to antibiotics that are widely used in human and veterinary medicine, including beta-lactams, tetracyclines, glycylcyclines, fluoroquinolones, aminoglycosides and sulfamethoxazole-trimethoprim, was analyzed by disk diffusion method. The prevalence of hemolysins, aerolysins (virulence factors) and integrase genes was determined. A total of 83 *Aeromonas* spp. strains were selected from the isolates obtained from river water and wastewater samples. The highest percentage (81.8%) of multidrug-resistant isolates was noted in DRW samples. The analyzed isolates were most frequently resistant to beta-lactams, tetracyclines and aminoglycosides, whereas resistance to glycylcyclines was least common. Isolates resistant to beta-lactams most frequently harbored bla_{TEM} and bla_{OXA} genes. The group of genes encoding resistance to tetracyclines was most frequently represented by *tet*(E) and *tet*(O). Genes encoding virulence *ahh*1 (heat-labile hemolysin) or integrase *int*I1 occurred more frequently in the strains isolated from DRW than URW. An analysis of genetic relatedness revealed two main clusters – one with closely related WW and DRW isolates and one with less related isolates from all analyzed samples. The results of this study indicate that regardless of the applied treatment, municipal sewage may be a reservoir of antibiotic-resistant bacteria, antibiotic resistance and virulence genes and that treated water can contaminate other environmental domains.

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1. Introduction

Antibiotics have been widely used in medical practice since the second half of the twentieth century. The overuse and misuse of antibiotics in human and veterinary medicine, animal husbandry, agriculture, aquaculture and food technology have contributed to an increase in antibiotic resistance and multidrug-resistance (MDR) of bacteria (Baquero et al., 2008; Harnisz, 2013). Several authors have suggested that antibiotic resistance in the clinical setting is closely associated with the mechanisms observed in environmental bacteria (Le Corre et al., 2012; Finley et al., 2013). In fact, aquatic ecosystems may provide an ideal setting for the acquisition and spread of antibiotic resistance because they are constantly exposed to anthropogenic changes such as pollution from urban, agricultural and industrial sources (Korzeniewska and Harnisz, 2013a, 2013b). Antibiotic-resistant bacteria reach aquatic ecosystems through surface runoffs, soil leaching and, above all, through effluents from municipal wastewater treatment plants (WWTP). WWTPs were originally designed to reduce biological oxygen demand, total suspended solids, nitrogen and phosphorus pollution, whereas the elimination of microorganisms was regarded as a less important goal (Lucas et al., 2014). Primary and secondary treatment processes remove up to 99% of fecal indicator bacteria (Korzeniewska and Harnisz, 2012; Korzeniewska and Harnisz, 2013a); however, the quality of treated wastewater may not be appropriate for irrigation or recreational activities in the receiving water bodies. These ecosystems may harbor microorganisms, including pathogens, which are resistant to antimicrobial agents and pose a threat to public health. This risk is exacerbated when bacteria are resistant to several groups of antibiotics at the same time (Sengupta et al., 2013). *Aeromonas* species frequently colonize freshwater systems, marine environments, soil, agricultural products and the digestive tract of fish and other aquatic animals (Korzeniewska et al., 2005; Pablos et al., 2009; Harnisz et al., 2015; Nawaz et al., 2006). They are often isolated from surface water, which can be attributed to high concentrations of total organic carbon and assimilable organic carbon. For this reason, *Aeromonas* species could be effective indicators of water pollution and water quality (Figueira et al., 2011; Percival et al., 2014). The most predominant *Aeromonas* species in surface water is *A. hydrophila,* closely followed by *A. caviae* and *A. veronii* biovar sobria. In view of their ubiquity and the harbored patterns of acquired antimicrobial resistance, members of the genus *Aeromonas* could be used to assess various types of antimicrobial resistance and their dissemination in different types of water bodies (Figueira et al., 2011). According to some authors (Janda and Abbott, 2010; Cattoir et al., 2008; Xia et al., 2010), there is a general scarcity of research into the low susceptibility of aeromonads to various classes and combinations of antimicrobial agents. The present study aims to fill this knowledge gap by hypothesizing that *Aeromonas* spp. from WWTPs could contribute to the dissemination of antibiotic resistance in surface water ecosystems.

Most *Aeromonas* spp. are generally harmful, and some of them could harbor virulence factors and cause life-threatening infections (Graevenitz, 2007; Aguilera-Arreola et al., 2005; Behera et al., 2011; Levine et al., 2010). *Aeromonas* spp. express a wide range of virulence factors, which enables them to colonize, invade and infect different hosts (Galindo et al., 2006). Cytolytic beta-hemolysin or aerolysin produced by different *Aeromonas* spp., including *A. hydrophila* and *A. veronii,* seems to be the most important enterotoxin (Rasmussen-Ivey et al., 2016). The extracellular heat-labile hemolysin (*ahh*1) is one of the most widely distributed hemolysins with the most cytotoxic genotype which is a synergistic combination of *aer*A and *ahh*1 genes (Wang et al., 2003).

There is evidence to indicate that antibiotic resistance and virulence factors are linked to bacterial populations (Zhang et al., 2015); therefore, the main goals of this study were to: (i) identify the key factors which contribute to the antibiotic resistance and virulence of mesophilic *Aeromonas* isolated from both river water and wastewater, and (ii) investigate the link between the origin of *Aeromonas* species and their antibiotic resistance and virulence pattern. Resistance to antibiotics that are most widely used in human and veterinary medicine (ECDC, European Centre for Disease Prevention and Control, 2015), including beta-lactams, tetracyclines, sulfonamides, glycylcyclines, aminoglycosides and fluoroquinolones, was analyzed in this study.

2. Materials and methods

2.1. Wastewater treatment plant

The investigated site was the Łyna Wastewater Treatment Plant in Olsztyn. The plant processes domestic effluents (175,000 inhabitants) as well as untreated sewage from the three hospitals. Hospital effluents generally account for less than 2% of the raw sewage processed by the WWTP (Korzeniewska et al., 2013). The plant's process line comprises mechanical, biological and chemical treatment sections, as well as sludge processing units. The plant has the following technical specifications: treatment system – activated sludge, average processing capacity – 60,000 m^3/d, wastewater type – municipal wastewater, mechanical treatment devices – screens, grit chamber and pre-sedimentation tank, biological treatment devices – separation chambers, aeration chambers and secondary sedimentation tanks, sedimentation devices – closed and open digestion chambers, belt filter press, incinerator. The hydraulic retention time (HRT) of wastewater is 24 h.

2.2. Sample collection

Samples of river water were collected upstream and downstream from the wastewater discharge point (URW and DRW, respectively) and samples of treated wastewater (WW) were collected from the secondary sedimentation tank. Samples of upstream river water and downstream river water were collected approximately 600 m from the discharge point of treated wastewater (N 53°49′7.27″, E 20°26′ 57.95″). Samples were collected on six occasions: in November 2014 and in January, April, June, July and August 2015 (a total of 18 samples). Water and wastewater samples were collected into sterile bottles, transported to the laboratory at a temperature of 4 °C and processed on the day of collection.

In our previous study (Harnisz, 2013) of water samples from the same sampling points, significant variations in dissolved oxygen concentrations and pH of URW, WW and DRW samples and an absence of variations in tetracycline levels (tetracycline, oxytetracycline, doxycycline) were observed across sampling sites.

2.3. Identification of Aeromonas spp.

The samples were diluted with saline solution (0.85% NaCl), and bacteria were cultured by the pour plate method on TSA medium (Tryptone Soya Agar, Oxoid) at a temperature of 37 °C for 24 h.

Colonies with various phenotypes were isolated from the TSA medium and purified on the same medium. Around 500 strains were isolated (100 from URW, 250 from WW and 150 from DRW). The strains were subjected to Gram-stain, glucose oxidation/fermentation, oxidase and catalase tests (Jacobs and Chenia, 2007). Straight coccobacillary to bacillary Gram-negative bacteria produce a fermentation reaction on the oxidation/fermentation basal medium, catalase- and oxidase-positive bacteria were chosen for further analyses as presumptive *Aeromonas* spp. A total of 123 isolates (21 from URW, 59 from WW and 43 from DRW) collected at different times were obtained. The strains were stored at −80 °C in the LB medium (Merck) with 10% addition of glycerol.

Genomic DNA was isolated by thermal lysis. A loopful of bacterial colonies harvested from a TSA plate was suspended in 0.5 mL of sterile water and heated at 95 °C for 10 min. After centrifugation at 5000 rpm for 5 min at 4 °C, the DNA-containing supernatant was used as the template for further amplification.