ANTIBIOTIC RESISTANCE PROFILING OF *AEROMONAS CAVIAE* STRAINS ISOLATED FROM WASTEWATER

Dr. Nowrotek Monika[1]
Jałowiecki Łukasz, M. Sc.[2]
Dr. Wasilkowski Daniel[3]
Prof. Płaza Grażyna[4]
[1, 2, 4]Institute for Ecology of Industrial Areas, Kossutha 6, 40-844 Katowice, Poland
[3]University of Silesia, Faculty of Biology and Environmental Protection, Jagiellonska 28, 40-032 Katowice, Poland

ABSTRACT

Resistance to various pollutants, including antibiotics, is widespread among environmental bacteria, including *Aeromonas* spp. The aim of the study was to investigate the antibiotic resistance pattern of *Aeromonas caviae* strains isolated from raw and treated wastewater taken from a municipal wastewater treatment plant.

The results from this study revealed high level of multidrug resistance among the tested isolates. Moreover, the strains showing greater resistance were found in raw wastewater (influent of wastewater treatment plants). The results indicate the impact of the applied wastewater treatment technology on the occurrence of resistance in *Aeromonas caviae*. There is a need for further study on the antibiotic resistance genes and genetic relatedness of these isolates.

Keywords: *Antibiotic resistance, Aeromonas caviae, wastewater*

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 [1]. Due to the unmonitored use of antibiotics and the release of residuals into the environment, widespread antibiotic resistance genes (ARGs) and emerging antibiotic-resistant bacteria (ARB) have become a great public concern. ARB and ARGs are recognized as emerging environmental pollutants. Effluents from urban wastewater treatment plants (WWTPs) are suspected to be among the main anthropogenic sources of antibiotics, ARB and ARGs spread into the environment. Horizontal gene transfer (HGT) is a major concern in understanding the spread of antibiotic resistance and developing strategies for mitigation. ARGs are often associated with mobile genetic elements (MGEs) including phages, plasmids, transposons and integrons [2], [3]. Among them, integrons are considered to be the most important contributors to MDR in bacteria and they also play a major role in disseminating antimicrobial resistance genes [4]. Bacteria that were initially susceptible to commonly used antibiotics,

currently they are becoming resistant. Among these antibiotic-resistant bacteria are some species of *Aeromonas*, both clinical and environmental isolates. Due to the ubiquitous nature of the members of *Aeromonadaceae*, they have also been linked to both food and water-borne diseases in different parts of the world [5]. *Aeromonas* species frequently colonize freshwater systems, marine environments, soil, agricultural products and the digestive tract of fish and other aquatic animals. It has been documented in different studies that members of the genus *Aeromonas* can harbor genes encoding beta-lactam and plasmid-mediated quinolone resistance with the potential to spread via horizontal gene transfer [6], [7]. This study aimed to investigate the antibiotic resistance pattern of *Aeromonas caviae* strains isolated from wastewater.

METHODOLOGY

Sample collection

The influent (raw wastewater – RW) and effluent (treated wastewater – TW) were collected from the municipal wastewater treatment plant (WWTP) located in the southern part of Poland (geographical coordinates: N 50° 5' 35.881; E 19° 3' 32.202). The samples were collected into 1 L sterile bottles and transported to a microbiology laboratory at 4°C using a cooler box. Bacterial isolation was carried out within 24-36 h after sample collection.

Selection and isolation of antibiotic resistant bacteria

Selection and isolation of bacterial strains were conducted using the spread plate technique. At the beginning of the experiment, 10^{-1} dilution of wastewater in 0,85% NaCl augmented with 6 combinations of antibiotics: (1) ofloxacin (16 mg L^{-1}) + norfloxacin (16 mg L^{-1}); (2) streptomycin (20 mg L^{-1}) + kanamycin (20 mg L^{-1}) + gentamycin (20 mg L^{-1}); (3) rifampicin (10 mg L^{-1}); (4) tetracycline (20 mg L^{-1}); (5) ofloxacin (16 mg L^{-1}); (6) norfloxacin (16 mg L^{-1}) was incubated at 30°C for 48 hours. Afterward, the diluted samples of wastewater were spread on solid Luria-Bertani (LB) medium amended with the same combinations of antibiotics. All the agar plates were incubated at 30°C for 24-72 hours. The number of colony forming units (CFU) of antibiotic-resistant bacteria was evaluated. In total, 38 and 36 bacterial strains were isolated from raw and treated wastewater, respectively.

Identification of antibiotic-resistant bacteria

The genomic DNA from 74 bacteria was isolated with Roche kit, according to the manufacturer's instruction. DNA was stored at −20°C until ready for use. The presence of integrons was detected by amplifying an internal fragment of integrase genes *int*I1 and *int*I2 according to the conditions described by Barraud et al., 2010 [8]. The class 1 integron was detected in all isolates, while class 2 integron was not detected in any isolate. Then, the isolates with i*nt*I1 were identified by 16S rRNA gene sequencing. Universal bacteria primers, 8F and 1492R were used to amplify 16S rRNA genes in accordance with the conditions established by Macrogen, Inc., Netherlands. All PCR amplifications were performed in a thermal cycler (Eppendorf). The obtained 16S rRNA sequence was compared with available

sequences in the Ezbiocloud. The phylogenetic tree of the analyzed isolates was built using Molecular Evolutionary Genetics Analysis software (MEGA7) [9].

Antibiotic susceptibility test

Antibiotic susceptibility was evaluated with a disk diffusion test as described elsewhere [10, 11]. The following antibiotics were used: amikacin (AMI: 30 μg L^{-1}), amoxicillin (AMO: 30 μg L^{-1}), ampicillin (AMP 25 μg L^{-1}), azithromycin (AZY 15 μg L^{-1}), aztreonam (AZT: 30 μg L^{-1}), cefaclor (CEF: 30 μg L^{-1}), cefadroxil (CFK: 30 μg L^{-1}), cefepime (CFP: 30 μg L^{-1}), cefoxitin (CFO: 30 μg L^{-1}), ceftaroline (CFR: 5 μg L^{-1}), ceftazidime (CFI: 30 μg L^{-1}), ciprofloxacin (CYP: 10 μg L^{-1}), doripenem (DRI: 10 μg L^{-1}), doxycycline (DOX: 30 μg L^{-1}), ertapenem (ERA: 10 μg L^{-1}), erythromycin (ERT: 30 μg L^{-1}), gentamycin (GNT1: 120, GNT2: 200), imipenem (IMP: 10), metronidazole (MET: 50 μg L^{-1}), minocycline (MNC: 30 μg L^{-1}), mupirocin (MUP: 200 μg L^{-1}), nalidixic acid (NAL: 30 μg L^{-1}), neomycin (NEO: 30 μg L^{-1}), netilmicin (NET: 30 μg L^{-1}), nitrofurantioin (NIT: 300 μg L^{-1}), norfloxacin (NOR: 10 μg L^{-1}), novobiocin (NOV: 30 μg L^{-1}), ofloxacin (OFL: 5 μg L^{-1}), piperacillin (PIP: 100 μg L^{-1}), rifampicin (RYF: 30 μg L^{-1}), teicoplanin (TEI: 30 μg L^{-1}), ticarcillin (TIC: 75 μg L^{-1}), trimethoprim (TRI: 5 μg L^{-1}), trimetoprim / sulfamethoxazole (TRI+SMX: 25 μg L^{-1}), tobramycin (TOB: 30 μg L^{-1}), vancomycin (VAN: 30 μg L^{-1}). The antibiotic disks were purchased from Oxoid. The bacterial strains were classified according to the inhibition zone diameter as sensitive (S) or resistant (R). The obtained results were performed according to EUCAST guidelines.

Determination of MAR (multiple antibiotic resistance) indexes

The MAR indices for the isolated bacterial strains were calculated following the formula as stated by Sair and Khan (2018) [12].

MAR index = number of antibiotics to which isolate showed resistance/number of total antibiotics exposed to the isolate

MAR index $\leq$ 0.2 is considered low risk, *e.g*. low or negligible antibiotic resistance; the isolate was never or hardly exposed to the tested antibiotic. MAR $\geq$ 0.2 indicates the high risk of antibiotic contamination; the isolate showed resistance to more than two of the tested antibiotics, and therefore distinct exposure to those antibiotics could be observed.

RESULTS

Based on the preliminary screening, 38 and 36 antibiotic-resistant strains were isolated from raw and treated wastewater, respectively. Among them, the *int*I1 gene was found in 24 strains from both raw and treated wastewater. The presence of class 1 integron in the final effluent is alarming, indicating that wastewater treatment plant is a reservoir for horizontal gene transfer for the selection of antimicrobial resistance genes among aquatic organisms in the environment. Consequently, wastewater treatment plant effluent discharges into the receiving water bodies pose a threat to the environment. On the basis of the conducted sequencing and bioinformatic analysis, 5 strains derived from raw wastewater (named RW_1, RW_2, RW_3, RW_4 and RW_5) and 11 strains derived from treated wastewater

(named TW_1, TW_2, TW_3, TW_4, TW_5, TW_6, TW_7, TW_8, TW_10, TW_11) were identified as *Aeromonas caviae.*

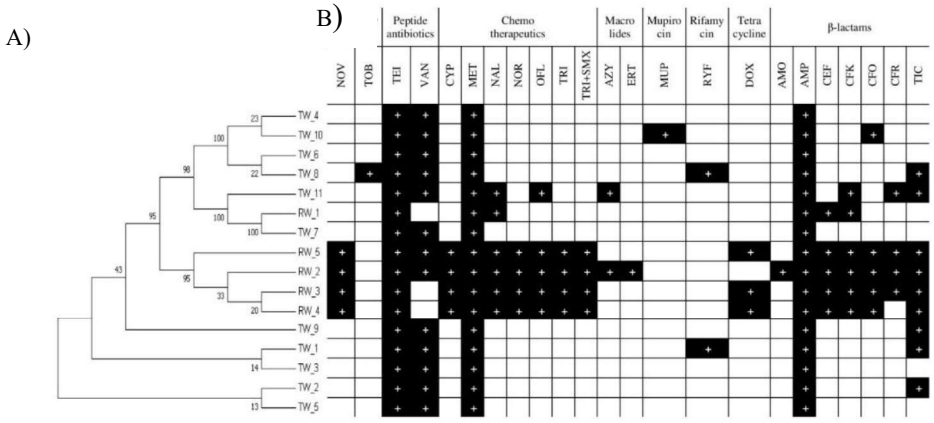

Figure 1: Neighbor-joining dendrogram based on the 16S rRNA nucleotide sequences of Aeromonas caviae (A) and characteristics of the isolates, regarding origin, antibiotic resistance profile (B)

Table 1: Results of antimicrobial susceptibility test for Aeromonas among EUCAST guidelines, where S: sensitivity, R: resistance

	TW_1	TW_2	TW_3	TW_4	TW_5	TW_6	TW_7	TW_8	TW_9	TW_10	TW_11	RW_1	RW_2	RW_3	RW_4	RW_5
aztreonam (AZT)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
cefepime (CFP)	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R
ceftazidime (CFI)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
ciprofloxacin (CYP)	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R
trimetoprim/ sulfamethoxazole (TRI+SMX)	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R

According to the classification of *Aeromonas* spp. with EUCAST guidelines, large variation within one species for selected antibiotics has been demonstrated (Table 1). Most strains isolated from treated wastewater show resistance to CFP, while strains isolated from raw wastewater were resistant to CFP, CYP and TRI + SMX.

All results, including phylogenetic analysis and antibiotic resistance profile of *Aeromonas caviae* are presented in Figure 1. The Figure 1A shows a rooted phylogenetic tree, in which 16 leaves can be distinguished, which correspond to the analyzed strains and 15 nodes indicating places below, whose objects do not have common features. Based on the bioinformatic analysis, no significant differences

were found between *Aeromonas* strains isolated from raw and treated wastewater. The two most similar objects were TW_2 and TW_5, characterized by the smallest distance between them. In the case of raw wastewater, two most similar objects were RW_3 and RW_4. Also, the similar antibiotic resistance profile was observed in these strains *e.g*. RW_3 and RW_4 as well as TW_2 and TW_5. The obtained results revealed high level of multi antibiotic resistance among the isolates. The isolates from raw wastewater showed resistance to a much higher number of antibiotics compared to isolates from treated wastewater. All tested strains were resistant to TEI, MET and AMP.

Additionally, the MAR index of all *Aeromonas* isolates ranged from 0.1 to 0.5 as seen in Figure 2 which reveals high level of use of antibiotics indiscriminately. Our results corroborate with other studies on antibiotic resistance of *Aeromonas* [13], [14]. Antibiotic resistance of *Aeromonas* species to multiple antibiotics is becoming a serious public health concern as revealed in this study.

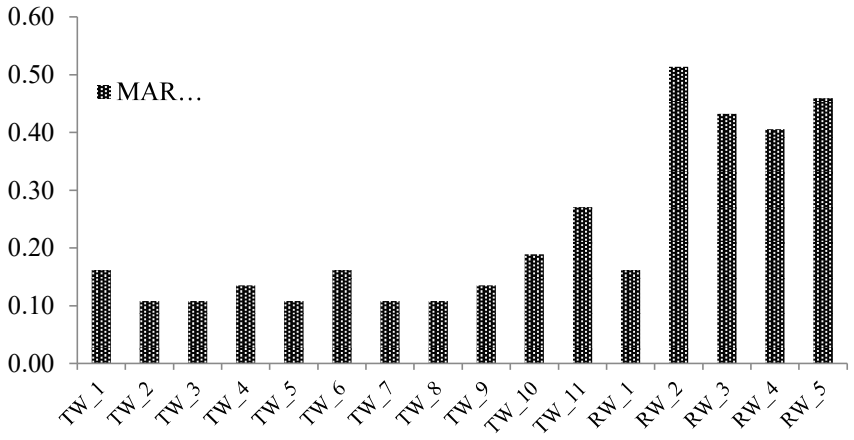

Figure 2: The value of MAR index calculated for isolated strains

CONCLUSION

The current findings show that *Aeromonas caviae* is a popular species in raw and treated wastewater. High incidences of antibiotic resistance were observed among the isolates. However, the resistance patterns differed among the tested strains of *Aeromonas caviae*.

Based on the marked MAR index, the antibiotic resistance of the tested *Aeromonas* species to the analyzed antibiotics was higher in raw wastewater as compared to strains from treated wastewater. It can be suggested that the technology used in wastewater treatment plants can indirectly affect the antibiotic resistance profile in bacteria. Moreover, phylogenetic analysis indicated on a lack of significant differences in genetic relationship between strains isolated from raw and treated wastewater. The results of this study confirms the presence of multi

antibiotic resistant *Aeromonas caviae* in wastewater, reflect popular occurrence of antibiotics and poses public health problem. Therefore, there is a need for further study on the antibiotic resistance genes and genetic relatedness of these isolates.

ACKNOWLEDGMENTS

This study was done under the project No 2017/26/M/NZ9/00071 funded by the National Science Centre, Poland.

REFERENCES

[1] Baquero F., Martinez J.L., Canton R. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. Vol. 19, pp. 260-265. 2008.

[2] Rizzo L, Manaia C., Merlin C., Schwartz T., Dagot C., Ploy M.C., Michel I., Fatta-Kassinos D. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci. Total Environ. Vol. 447, pp. 345-360. 2013.

[3] Zhang T., Zhang X., Ye L. Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge PLoS One, Vol. 6/Issue 10, Article e26041. 2011.

[4] Krauland M.G., Marsh J.W., Paterson D.L., Harrison L.H. Integron – mediated multidrug resistance in a global collection of nontyphoidal *Salmonella enterica* isolates. Emerg. Infect. Dis., Vol. 15/issue 3, pp. 388-396. 2009.

[5] Levine S.M., Frangos S.G., Hanna B., Colen K., Levine J.P. *Aeromonas* septicemia after medicinal leech use following replantation of severed digits. Am. J. Crit. Care. Vol. 19/issue 5, pp. 469-471. 2010.

[6] Cattoir V., Poirel L., Aubert C., Soussy J., Nordmann P. Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp.

Emerg. Infect. Dis. Vol. 14, pp. 231-237. 2008

[7] Moura A., Oliveira C., Heinriques I., Smalla K., Correia A. Broad diversity of conjugative plasmids in integron-carrying bacteria from wastewater environments FEMS Microbiol. Lett. Vol. 330, pp. 157-164. 2012.

[8] Barraud O, Baclet M.C., Denis F., Ploy M.C. Quantitative multiplex real – time PCR for detecting class 1, 2 and 3 integrons. J Antimicrob Chemother. Vol. 65/issue 8, pp 1642 – 1645. 2010.

[9] Kumar S., Stecher G., Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. Vol. 33, pp. 1870-1874. 2016.

[10] Bauer A. W., Kirby W. M., Sherris J. C., Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. Vol. 45/issue 4, pp. 493–496. 1966.

[11] Clinical and Laboratory Standards Institute (CLSI), Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically: Approved Standard M7-A7, Clinical and Laboratory Standards Institute, Wayne, Pa, USA, 7th edition, 2006.

[12] Sair A.T., Khan Z.A. Prevalence of antibiotic and heavy metal resistance in gram negative bacteria isolated from rivers in northern Pakistan. Water and Environmental Journal. Vol. 32, pp. 51-57. 2018.

[13] Carnelli A., Mauri F., Demarta A. Characterization of genetic determinants involved in antibiotic resistance in Aeromonas spp. and fecal coliforms isolated from different aquatic environments. Research in Microbiology. Vol. 168/Issue 5, pp. 461-471. 2017.

[14] Piotrowska M., Przygodzińska D., Matyjewicz K., Popowska M. Occurence and variety of β-lactamase genes among Aeromonas spp. isolated from urban wastewater treatment plant. Front. Microbiol. Vol. 8, article number 863. 2017.