The prevalence of virulence genes specific for *Escherichia coli* in wastewater samples from wastewater treatment plants with the activated sludge process

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Abstract. Treated wastewater evacuated into the aquatic environment is a potential reservoir of pathogenic and virulent bacteria. The aim of this study was to analyze the presence of genes encoding virulence in *E. coli* bacteria in samples of untreated (UWW) and treated (TWW) wastewater from 13 wastewater treatment plants deploying various sewage treatment methods. Wastewater samples were passed through polycarbonate membrane filters, and genomic DNA was extracted. Virulence genes specific for *E. coli* were detected by standard PCR and were grouped according to their association with different pathotypes. The *stx*2 gene was most prevalent in samples of UWW, and the *eae* gene was most frequently detected in samples of TWW. An analysis of virulence markers revealed a predominance of genes characteristic of STEC and EIEC pathotypes. The highest variability of virulence genes was observed in wastewater treatment plants where sewage is treated mechanically and biologically, and the lowest variability was noted in plants deploying the A2/O treatment process (3-stage Bardenpho). In several plants, the prevalence of virulence genes increased after treatment. The results of this study suggest that wastewater treatment plants are significant reservoirs of virulent bacteria. The evacuation of TWW into water bodies can contribute to the dissemination of virulence genes in the environment, which poses a serious health hazard for humans and animals.

1 Introduction

Wastewater treatment plants (WWTPs) are major reservoirs of bacteria which are transported to the aquatic environment with treated sewage. The operating conditions inside wastewater treatment plants, including high nutrient load, optimal temperature and high microbial concentrations, create supportive conditions for the transfer of genes between bacteria [1]. Up to 99% of bacteria are eliminated during wastewater treatment [2,3], but considerable numbers of bacteria, including bacteria that harbor drug-resistance and virulence genes, are discharged into the aquatic environment with treated wastewater [4].

The main wastewater components are human feces whose quantity and composition determine bacterial loads in sewage [5]. The dissemination of gut bacteria, including pathogenic and potentially pathogenic bacteria, in the natural environment can have

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negative implications for human and animal health [6]. Those risks are exacerbated by bacteria which are resistant to antibiotics and harbor virulence genes.

Escherichia coli is part of typical gut microbiota in humans and animals, and it can be transmitted to the environment directly with feces or indirectly with treated wastewater. *Escherichia coli* is an important indicator of fecal contamination of various environments and food [2, 6]. According to many authors, the presence of *E. coli* should be monitored to evaluate the microbiological quality of wastewater and contamination of aquatic ecosystems [7]. Most *E. coli* strains are commensal bacteria, but some strains are pathogenic and harbor specific virulence factors responsible for intestinal and non-intestinal infections, urinary tract infections, meningitis and sepsis [8].

Escherichia coli strains harboring virulence genes are the most frequent cause of urinary tract infections [9]. They are detected mainly in hospitals and are indirectly introduced to the aquatic environment with hospital wastewater [3]. The virulence of *E. coli* bacteria is determined mainly by virulence factors which are localized on mobile genetic elements and spread by horizontal gene transfer (HGT) [10]. Virulence factors determine bacteria's ability to survive in diverse environments. Environmental changes stimulate the development of new combinations of virulence genes and the dissemination of new and more virulent strains [11]. *Escherichia coli* can be classified into various pathogen groups based on the presence of different virulence genes. Virulent *E. coli* strains pose a growing health threat and a considerable challenge for contemporary medicine. Strains that harbor virulence genes are often highly resistant to antibiotics, and infections caused by these bacteria are increasingly difficult to treat [12].

The virulence mechanisms in *E. coli* are genetically encoded by chromosomal or plasmid DNA or their spread is mediated by bacteriophages. At least 567 virulence genes (VGs) have been identified in *E. coli*. They can be divided into 78 groups of virulence factors (VFs) with various functions, including adhesins, toxins, capsules, secretion systems, iron uptake systems and invasins [13,14]. The virulence markers selected for the study are represented by the following genes: *eae* (attaching and effacing lesions, intimin encoding gene), *bfp*A (localized adherence, encoding the production of type IV pili), the CVD432 gene encoding proteins responsible for enteroaggregative adherence, *ipa*H (enteroinvasive mechanism, responsible for adhesion to and colonization of epithelial cells), the LT*gene* encoding heat-labile toxin (activates adenylyl cyclase on the surface of epithelial cells and disrupts ion pump function), the heat-stable toxin ST *gene* (activates guanylyl or adenylyl cyclase on the cell surface and induces ion outflow from cells), *stx*1 and *stx*2 (Shiga toxins, inhibit protein synthesis and induce cell apoptosis) [8, 15].

Based on their virulence properties and clinical manifestations in the host, pathogenic *E. coli* isolates can be divided into two main groups: intestinal pathogenic *E. coli* (IPEC) and pathogenic *E. coli* parenterally (ExPEC). The first major group of pathogenic *E. coli* causes symptoms characteristic of gastrointestinal disease, and it consists of pathotypes of enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC) and a subgroup of enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusively adhesive *E. coli* (DAEC) [16, 17]. The second major group of pathogenic *E. coli* comprises extraintestinal pathogenic *E. coli* (ExPEC), and it includes avian pathogenic *E. coli* (APEC) which cause respiratory tract infections and septicemia in poultry and uropathogenic *E. coli* (UPEC) [9]. The virulence mechanisms in various pathotypes of *E. coli* are genetically encoded by chromosomal, plasmid and bacteriophage DNA. The presence of specific virulence markers correlate, sometimes very closely, with the pathotype of diarrheagenic *E. coli* [8,18].

The aim of this study was to determine the prevalence of virulence genes in *E. coli* bacteria in samples of untreated (UWW) and treated wastewater (TWW) from different

wastewater treatment plants. The results can be used to evaluate the potential risks for the natural environment and human health.

2. Materials and methods

2.1. Sampling sites and samples collection

Samples of untreated and treated sewage was collected from 13 WWTPs located in Warmia and Mazury District. WWTPs treat the wastewater based on activated sludge with different modification (biological, biological with elevated nutrient removal) and type of inflowing wastewater (domestic sewage, hospital sewage, wastewater from food industry), various capacity and number of population served. Wastewater treatment plant were divided into 4 categories, due to the applied modification of sewage treatment system (Tab. 1). Samples of wastewater were collected into the sterile bottles, transported to the laboratory at the temperature of 4ºC and processed on the day of collection.

Table 1. Division of wastewater treatment plants from which samples were taken.

The number of treatment plan	Type of sewage treatment technology used	Type of incoming effluent
I	A. WWTPs with A_2O system,	domestic sewage
X		domestic sewage +wastewater from food industry
II	B. WWTPs with mechanical-biological system	domestic sewage +hospital sewage +wastewater from food industry
IV		domestic sewage
VII		domestic sewage +wastewater from food industry
VIII		domestic sewage +wastewater from food industry
IX		domestic sewage
III	C. WWTPs with Sequencing Batch Reactors (SBR)	domestic sewage
VI		domestic sewage
XI		domestic sewage
V	D. WWTPs with mechanical-biological system with elevated removal of nutrients	domestic sewage
XII		domestic sewage +hospital sewage +wastewater from food industry
XIII		domestic sewage +hospital sewage +wastewater from food industry

2.2. DNA extraction

Wastewater samples (1000 mL of untreated wastewater and 2000 mL of treated wastewater) were passed through polycarbonate filters with a diameter of 47 mm and a pore size of 0.2µm (Merck, Millipore). Then, DNA extraction filters were transferred to a sterile screw cap tubes (50 mL), and 30 mL of 1 x PBS was added to the tubes. They was shaken (200 rpm/min, 3 hours) at room temperature. Then entire precipitate was transferred to 2.0 mL Eppendorf tubes and centrifuged (9000 rpm/min, 15 min). After centrifugation, the entire precipitate was transferred to 2.0 mL Eppendorf tubes [19]. Then DNA extraction was performed using isolation kits (Genomic Mini A&A Biotechnology kit) according to the manufacturer's instructions. The concentration and quality of extracted DNA was

determined by microspectrophotometry (NanoDrop® ND-1000, Nano Drop Technologies, Willmington, DE). DNA was stored at −20°C for further analysis.

2.3. Analysis of the prevalence of virulence genes

The obtained DNA was used to perform qualitative analysis of eight genes responsible for virulence. The presence of virulence genes has been determined based on standard PCR reaction which detects typical and atypical enteropathogenic *E. coli* isolates (EPEC: *bfp*A and *eae* genes), enteroaggregative *E. coli* isolates (EAEC: CVD43 gene), enterotoxigenic *E. coli* isolates (ETEC: LT and ST genes), entero-invasive *E. coli* isolates (EIEC: *ipa*H gene) and Shiga toxin producing *E. coli* isolates (STEC: *stx*1 and *stx*2 genes) [17].

The reactions were carried out in a 20 µl mixture containing: 2 µl PCR bufor, 8 µl nuclease free water, 0.5 µl (10 µM concentration) of each primers, 5 µl polymerase (1U), 1 µl nucleotide mix (10 µM concentration) and 1 µl DNA template. The PCR reactions conditions were made according to Aranda et al. [20].

3. Results and discussion

Faeces are the main source of pathogenic microorganisms, including bacteria of the genus *Escherichia coli*, in wastewater. Treatment processes reduce the concentrations of pollutants, including microbiological contaminants, in wastewater, but large quantities of potentially pathogenic bacteria are evacuated to water bodies [4]. The transfer of virulence genes between bacteria increases their pathogenic potential and exacerbates the relevant health risks for humans and animals.

The most prevalent virulence gene in all analyzed wastewater samples, regardless of the evaluated groups of wastewater treatment plants or treatment stage (Fig. 1), was *stx*2(23%), followed by *ipaH* (20%) and *eae* (18%). The least frequently encountered genes were *bfp*A, CVD432 and *stx*1. In a study analyzing *E. coli* in wastewater samples obtained from a plant with a mechanical and biological treatment system and enhanced removal of biogenic compounds, the most prevalent genes were *bfp*A (65%), ST (56%) and *eae* (39%) [12]. In the work of Koczura et al. [21], the *stx*1 gene was least frequently identified in wastewater.

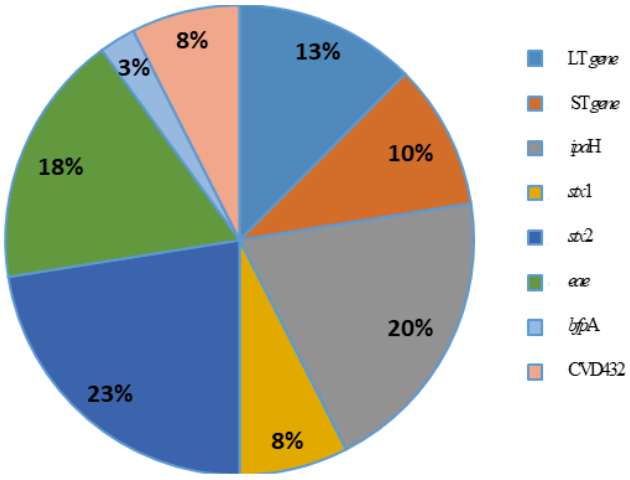

Fig. 1. General prevalence of virulence genes in wastewater.

When the treatment stage was taken into account in analyses, *stx*2 gene was the most prevalent in UWW samples, and the *eae* gene was the most frequently detected in TWW

samples (Fig. 2). According to Ateba et al. [15], the high prevalence of the *eae* gene could be attributed to the presence of clinical strains from individuals with diarrhea. It should also be noted that the frequency of occurrence of LT, ST, *eae* and *bfp*A genes increased after treatment. Interestingly, the *bfp*A gene was not detected in UWW samples. The frequency of occurrence of the remaining virulence genes decreased after treatment. Similar results were reported by Firgon et al. [4], who concluded that selected gene functions could be lost or driven out by other genes during wastewater treatment. The latter case could arise because these genes are associated with each other and because the function of one of the genes is counter-selected, or they are carried together on unstable mobile genetic elements such as pathogenicity islands (PAIs), plasmids, or prophages.

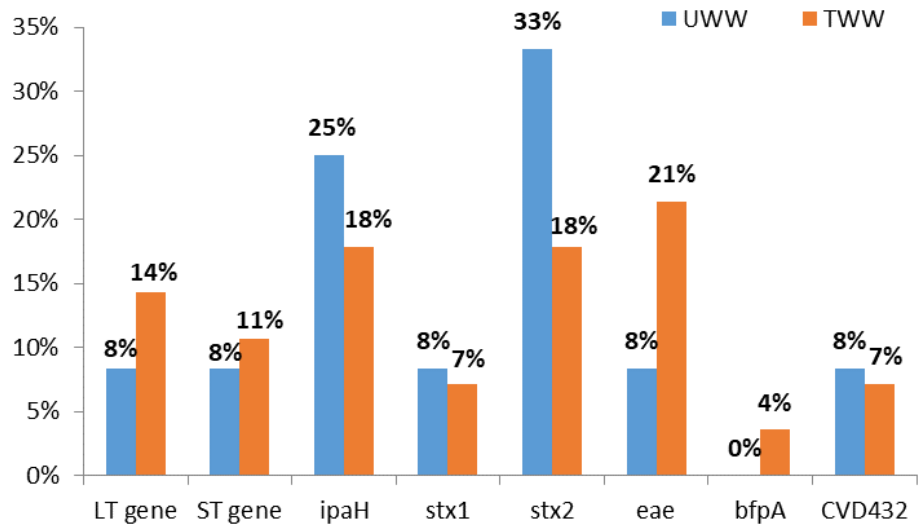

Fig. 2. Prevalence of virulence genes in different stages of wastewater treatment.

In the current study, genes characteristic of all *E. coli* pathotypes were detected in all WWTPs with mechanical and biological treatment systems (group B plants). These results indicate that TWW discharged to the aquatic ecosystems could pose a serious health hazard (Table 2).

Table 2. Prevalence of virulence genes in the analyzed groups of wastewater treatment plants.

Group	Stage	ETEC		EIEC	STEC		EPEC		EAEC
		LT *gene*	ST *gene*	*ipa*H	*stx*1	*stx*2	*eae*	*bfp*A	CVD432
A	UWW	-	-	+	-	-	-	-	-
	TWW	-	-	-	+	-	-	-	-
B	UWW	+	-	+	-	+	+	-	+
	TWW	+	-	+	+	+	+	+	+
C	UWW	-	+	+	-	+	-	-	-
	TWW	+	+	+	-	+	+	-	-
D	UWW	-	-	-	+	+	-	-	-
	TWW	+	+	+	-	+	+	-	+

Virulence genes and *E. coli* pathotypes were least prevalent in TWW samples from WWTPs with mechanical-biological-chemical treatment systems with the A2/O process

(group A plants). An analysis of virulence markers revealed that STEC and EIEC were the most frequent pathotypes. Similar results were obtained by Firgon et al. [4], who stated that STEC was also the most prevalent pathotype in wastewater samples.

4. Conclusion

The results of this study indicate that wastewater treatment plants are major reservoirs of potentially pathogenic bacteria harboring virulence genes. The prevalence of the investigated virulence genes differed between WWTPs deploying various treatment methods. In some WWTPs, the prevalence of virulence genes did not decrease after treatment. These findings indicate that wastewater treatment processes and the release of TWW into the aquatic environment should be closely monitored to eliminate potential health risks for humans and animals.

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