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Indication of Phages and Their Role in the Regulation of the Purification Process at the Govsany Aeration Station in Baku

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Abstract. Due to the epidemic situation and the emergence of enteroviral diseases, we set ourselves the task of conducting comprehensive virological and bacteriological studies of the wastewater, its sludge and contaminated soil from the Govsany aeration station. The fact of constant detection of enteroviruses in samples of wastewater and soil in combination with bacteriophages is considered by us as a possibility of detection of bacteriophages at WWTP sites. The 26 wastewater samples contain enteroviruses at various stages of treatment, 18 of which were characterised by high levels of faecal contamination (thermoletarian coliforms), *C. perfringens* and *Enterococcus faecalis*. In samples with high contamination (TCB up to 106 per 100 ml of wastewater), which are potentially dangerous in terms of the content of infectious disease pathogens, both bacterial and viral, the number of coliphages was below the norm. Consequently, coliphages cannot be used as a sanitary indicator of microorganisms of viral contamination. Intestinal phages are common where humans and animals live and therefore intestinal bacteria are present. Coliphages (bacteriophages) can be formed from lysogenic enterobacteria during the process of stepwise wastewater treatment at the Govsany WWTP.

Key words: enteroviruses, soil, wastewater, sludge, bacteriophages, anaerobic organisms, Cl sporogenes, indication, activated sludge plant

Introduction

The virological and bacteriological control of waste water and its sludge, together with the study of soils, is very topical.

It should be taken into account that the discharge of wastewater (domestic, agricultural, industrial) creates a direct epidemiological danger in the area of its release due to the bacterial background and a large number of easily oxidised organic compounds, which are the energy base for the additional development of microorganisms (Vorobyova et al., 2011; Litvin & Pushkareva, 1994).

The eutrophication of natural water systems causes a number of serious sanitary and hygienic problems in the areas of drinking and recreational water use, creates a nutrient base for the development of pathogenic flora and viruses, and poses a real and potential threat to the health of the population (Markova et al., 2009; Sherbo, 2011).

Today, enteroviruses are the only group of viruses for which reliable methods have been developed to detect them in water and soil and to confirm infectivity (Shah Mahmud et al., 2013).

For many years, we have been conducting sanitary-bacteriological and virological studies of soil samples and wastewater after various types of purification from wastewater treatment plants in the city of Baku. In 2008-2010, 330 wastewater samples were tested for bacterial contamination and 496 wastewater samples were tested for 83 viral strains. It should be noted that the spectrum of isolated enteroviruses reflected the spectrum of enteroviruses circulating among the population of Baku at that time (Sadikhova, Gulieva, & Agabekova, 2012; Sadikhova et al., 2012; Guliyeva, 2012). At present, in 2023, we have set ourselves the task of conducting new comprehensive virological and bacteriological studies of wastewater, its sludge and contaminated soil from the Govsany aeration station, while studying the

epidemiological situation and the occurrence of enteroviral diseases. The fact that enteroviruses have been consistently detected in soil and water samples in combination with bacteriophages is seen by us as a possibility to detect bacteriophages at sewage treatment plant sites. Extensive research into bacteriophages has led to their widespread use in solving many problems in microbiology, virology, immunology and biotechnology. As a result, the study of phages has now acquired general biological significance. Based on the above, the mechanism of involvement of the phage population in the treatment of wastewater and soils adjacent to pool cleaning is very interesting. Therefore, the aim of this work was to carry out comprehensive studies of wastewater, sludge, soil, indication of phages and their role in the regulation of the purification process at the Govsany Aeration Treatment Plant in Baku.

Materials and Methods

Soil analysis was carried out to determine bacteria, their types and numbers. Soil samples were taken from different locations in the Govsany aeration station. The samples were taken with a sterile spatula from a depth of 10-20 cm. The samples were cleaned of various impurities (roots, stems, leaves) and then soaked in sterile spring water for 15 minutes. The samples were then stored at room temperature (20-22°C) for 5 days. A small amount of soil sample was inoculated on a Petri dish with MPA (meat peptone agar) using a microbiological loop. The dishes with the cultures were then kept on a worktable in a box for 5 days, the growth of the cultures was observed, then smears were made, stained with Gram and examined under the microscope.

The microbiological analysis of the effluent was carried out according to MU2.1.5.800-99. The number of coliform bacteria, shigella, salmonella, streptococci, pneumococci, clostridii, bacilli and moulds of the genus Aspergillus spp. were determined. A common characteristic of wastewater after all types of treatment is the possibility of contamination by pathogenic or opportunistic microflora, as well as viruses, which is particularly relevant today.

The total microbial count was determined by the number of microorganisms in 1 ml of effluent. After the subsequent purification step, different dilutions (104-106) of each sample were inoculated in a volume of 1 ml into sterile Petri dishes and the sample was poured over with melted and cooled to 45°C meat peptone agar and the contents of the dishes were mixed evenly, avoiding the formation of bubbles. After solidification, it was placed in a thermostat and incubated at 37°C for 24 hours. General and thermotolerant bacteria were determined by membrane filtration. A measured volume of effluent was passed through a membrane filter using a PVF-35B vacuum filtration device, then the filter was transferred to the Endo medium without turning, ensuring complete adhesion to the nutrient medium. The dishes were incubated in a thermostat at 37°C for 24 hours.

Selective and differential diagnostic media were used to isolate and identify microorganisms found in the effluent. Blood agar and glucose agar were used for inoculation to isolate members of the genus Streptococcus. Microorganisms of the Enterobacteriacea family were identified using API-20 E tests (Biomerieux).

Species composition of microorganisms detected in the effluent of the Govsany aeration plant: Escherichia coli, E. coli, Streptococcus, Shigella, Staphylococcus, Bacillus, Enterococcus, Pseudomonas, Clostridii, Aspergillus spp. (Table 1).

Results and Discussion

Of the 26 wastewater samples containing enteroviruses at various stages of treatment, 18 samples were characterized by high levels of fecal contamination (thermoletarian coliforms), as well as C. perfringens. In 7 samples, coliphages were detected up to 10^{-5} PFU (plaque-forming units) for every 100 ml of wastewater. At the same time after biological treatment in one sample of waste water, viruses were isolated with a low number of coliform bacteria - 10

CFU per 100 units and a coliphage content of 50 PFU per 100 ml of wastewater; just these cases are put forward in favor of the indicator value of coliphages and their advantages compared to bacterial indicators.

In samples with severe fecal contamination (TBC up to 10^6 CFU per 100 ml of wastewater), that is potentially dangerous in terms of the content of pathogens of infectious diseases of both bacterial and viral nature, the number of coliphages has been recorded below the norm to date. We also found that coliphages are contained in feces in small quantities. Consequently, coliphages cannot play the role of sanitary indicator microorganisms of viral contamination, and the detection of coliphages in wastewater in the absence of them in feces indicates different ways of entry of coliphages and viruses into water. Guided by MU 2.1.5.800-99, MUK 4.2.1018-01, we identified not only coliphages, but a significant part of bacteriophages of enterobacteria. In this case, lysogenic enterobacteria can be a source of the appearance of phages in water, which were determined to be coliphages during the analysis.

In our wastewater analyses, the calculated concentration of Enterobacteriaceae was 10^5 CFU/L. The samples which were prepared in this way obviously did not contain free phage. But during the research, phages were isolated in 3 out of 7 cases in accordance with MUK4.2.1018-01. Here you need to pay attention to the fact that, according to the recommendation of MUK, titration method provides inoculating the test wastewater sample into a nutrient broth Triptone Water with E.coli. (Liofilchem Italy) undoubtedly, in this case, microorganisms not only of the introduced detector strain will multiply, but also bacteria initially contained in the wastewater after mechanical treatment, and their number can even reach 10^8 CFU/ml.

Accordingly, the likelihood of obtaining a false positive result increases. It is obvious that lysogenic enterobacteria, including E. coli, are present in water, and containing a moderate amount of phages in them can spontaneously create high concentrations of phages in water under the influence of various chemical or physical factors.

Table 1. Spectrum of microorganisms circulating in wastewater in autumn-winter period in Baku

Microorganisms	**Govsany aeration drainage station**											
	Before mechanical cleaning			**After mechanical cleaning**			**Biological cleaning**			**Activated sludge**		
	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-6}
E. coli	+	+	+	+	+	+	+	-	-	+	+	-
Shigella	+	+	+	+	+	+	+	-	-	+	-	-
Enterococcus faecalis	+	+	+	+	+	+	+	+	+	+	+	-
Staphylococcus	+	+	+	+	+	+	-	-	-	+	-	-
Klebsiella pneumonia	+	+	+	+	+	+	+	+	-	+	-	-
Streptococcus pneumonia	+	+	+	+	+	+	+	+	-	+	+	-
Pseudomonas	+	+	+	+	+	-	+	+	-	+	+	-
Cl. perfringens	+	+	+	+	+	+	+	-	-	+	+	-
Bacillus spp	+	+	+	+	+	+	-	-	-	+	-	-
Aspergillus spp	+	+	+	+	+	+	+	+	+	+	+	+

Soils taken from a wastewater treatment plant after stepwise treatment were dominated by fungi, actinomycetes and anaerobic bacteria (Cl. perfringens and Cl. sporogenes) in the lower layers. Shigella, Escherichia coli and other aerobes were present in the top layer. Cl. perfringens and Cl. sporogenes were very interesting, forming colonies of irregular shape on blood agar, grey, 3-6 x 0.5 microns in size, convex and with a significant lysis zone, as well as Enterococcus faecalis. For further study, the zones of bacterial lysis marked on the dishes were excised with a sterile lancet and placed in a sterile tube, to which 5 ml of liquid MPA broth was added, enriched and incubated for 24 hours. The resulting mixture was then centrifuged at 5000 rpm for 15 minutes, the supernatant passed through a 0.2 μm pore filter and tested for the presence of phages using the agar layer method. For this purpose, 0.1 ml of the test suspension and 0.2 ml of a sensitive bacterial culture were mixed and added to 3 ml of 0.7% MPA agar, melted and cooled to 45-50°C, mixed and spread over the surface with 1.5% agar. The cultures were then incubated for 48 hours at an optimum temperature of 30-40°C and the appearance of negative phage was analysed for bacterial lawn. The morphology of negative colonies was examined during inoculation using agar layers. Negative colonies had a similar morphology: round shape with smooth edges, transparent, diameter from 2.5 to 4 mm, lytic activity up to 10 according to the Appelman method. The bacteriophages had a pronounced specificity for bacteria of the genus Enterococcus and were heat-labile and chloroform-resistant. Thus, we identified specific phages from samples of contaminated soil at the Govsany aeration station that lysed bacteria of the genus Enterococcus pathogenic to humans and animals.

Conclusions

1. Phages have an enormous reproductive capacity, resulting in the release of individual virus particles from a cell within a short time after infection. Thousands of viral particles can be produced in a single bacterial cell; such fecundity is often accompanied by lysis.

2. Phages of intestinal bacteria are common where humans and animals live, and so are intestinal bacteria.

3. Coliphages (bacteriophages) can be formed from lysogenic enterobacteria during wastewater treatment in the Hovsany aeration plant.

4. The data obtained allow the use of specific phages for the detection and identification of homologous bacteria.

5. The detection of enteroviruses even after treatment with chlorination at a dose sufficient to inactivate bacterial microorganisms E. coli indicates the importance of using more effective measures for disinfection of wastewater and sludge deposits, not only with regard to enteroviruses but also with regard to anaerobic microorganisms.

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