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Investigation of SARS-CoV-2 and Gastrointestinal Pathogens in a Municipal Wastewater Treatment Plant in Turkey

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Abstract

In this study, SARS-CoV-2 and gastrointestinal pathogens in a municipal wastewater treatment plant (MWWTP) in Elazığ (Turkey) were investigated. The gastrointestinal pathogen analyzes were performed in influent and effluent of MWWTP, and SARS-CoV-2 analyzes were performed in different treatment units. According to obtained analysis results, gastrointestinal pathogens (bacterial, viral, EPA, and stool parasites) were detected in influent of the MWWTP. Enterohemorragic/verotoxin-producing *Ecsherichia coli*, all viral agents causing gastroenteritis (except sapoviruses (Sapo)), human parechovirus (HPeV) and adenoviruses from EPA, and *Giardia lamblia* from stool parasites were detected in effluents of the MWWTP. When bacterial agents causing gastroenteritis, viral agents, EPA, and stool parasites were investigated in the effluent of MWWTP, only 1 agent out of 7, 4 agents out of 6, 2 agents out of 3, and 1 agent out of 3 was detected as positive (+), respectively. SARS-CoV-2 could not be detected in the samples taken from each unit of the wastewater treatment plant. As a result, according to research findings, since pathogens are encountered in wastewater treatment plant effluents, it is necessary to have a disinfection system in the treatment plants and to 500onitör pathogens and SARS-CoV-2 continuously in order to protect environmental and human health.

Keywords: Gastrointestinal, pathogen, SARS-CoV-2, treatment, viruses, wastewater

Türkiye'de Kentsel Bir Atıksu Arıtma Tesisinde SARS-CoV-2 ve Gastrointestinal Patojenlerin Araştırılması

Öz

Bu çalışmada, Elazığ'da (Türkiye) bir kentsel atıksu arıtma tesisinde (AAT) SARS-CoV-2 ve gastrointestinal patojenler araştırılmıştır. Gastrointestinal patojen analizleri AAT giriş ve çıkış sularında, SARS-CoV-2 analizleri ise arıtma tesisinin farklı ünitelerinde yapılmıştır. Elde edilen analiz sonuçlarına göre AAT'nin girişinde gastrointestinal patojenler (bakteriyel, viral, EPA ve dışkı parazitleri) tespit edilmiştir. AAT'nin atıksularında enterohemorragik / verotoksin üreten *Esherichia coli*, gastroenterite neden olan tüm viral ajanlar (sapovirüsler (Sapo) hariç), insan parekovirüs (HpeV) ve EPA'dan adenovirüsler ve dışkı parazitlerinden *Giardia lamblia* tespit edilmiştir. AAT çıkışında gastroenterite neden olan bakteriyel ajanlar, viral ajanlar, EPA ve dışkı parazitleri incelendiğinde 7'de sadece 1, 6'da 4, 3'te 2 ve 3'te 1 ajan pozitif (+) olarak tespit edilmiştir. Atık su arıtma tesisinin her bir ünitesinden alınan numunelerde SARS-CoV-2 tespit edilememiştir. Sonuç olarak, araştırma bulgularına göre, atık su arıtma tesisi atık sularında patojenlerle karşılaşıldığından, çevre ve insan sağlığını korumak için arıtma tesislerinde dezenfeksiyon sistemine sahip olmak ve patojenleri ve SARS-CoV-2'yi sürekli izlemek gerekmektedir.

Anahtar Kelimeler: Gastrointestinal, patojen, SARS-CoV-2, arıtma, virüsler, atıksu

INTRODUCTION

The World Health Organization predicts that globally about 10% of diseases can be attributed to water quality and related hygienics issues (Prüss-Üstün et al., 2008). Furthermore, water pollution has resulted in deaths of 100 million human, 100,000 sea mammals, and 1 million seabirds per year according

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to World Water Assessment Programme (2003-2016) and United Nations Education, Scientific, and Cultural Organization) (Kumar et al., 2018). The main source of pollution in water bodies is the discharge of wastewater (Xu et al., 2018). Wastewaters may contain various pollutants such as heavy metals (toxic pollutants that are non-biodegradable in the ecosystem and can accumulate in the food chain) and pathogens (Ahmad et al., 2015; Topal and Arslan Topal, 2017; Arslan Topal and Elitok, 2018) Effluents of wastewater treatment plants (WWTPs) are important for public health if they are given into the water bodies to be used for drinking, recreation or agricultural purposes. Because, urban wastewaters contribute to infectious diseases (Xiao et al., 2018). Among pollutants in wastewater, pathogens are a source of concern because of their ability to cause diseases (Arora and Kazmi, 2015) including lethal ones (e.g., reactive arthritis, diabetes, myocarditis, and cancers) (Ashbolt, 2004; Kumar et al., 2018).

The potential pathogens present in wastewater are enterovirus, bacteria, rotavirus, protozoa, and helminth eggs (Verlicchi and Zambello, 2015; Marin et al., 2015). In year 2019, novel coronavirus (SARS-CoV-2) was added to these pathogens. Viruses and microorganisms can cause the risk for human health, environmental pollution and economic impacts. Not adequately treated wastewater can lead to persistence of these viruses and microorganisms in the environment (Barrett et al., 2016). Furthermore, pathogenic microorganisms via aeration and mechanical mixing can be released to the atmosphere and can lead to potential health risks in workers of WWTP (Xu et al., 2018).

SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) causes COVID-19. The disease includes both pauci-symptomatic or asymptomatic forms and quickly progressive deadly forms (Vespa et al., 2021). Over 90 million cases are confirmed worldwide as of early January 2020 resulting in pandemic (Ballow and Haga, 2021). Before COVID-19 pandemic, published studies have proved that coronaviruses can be shed in the feces. Also in recent studies, viral RNA has been identified in samples of stool from infected patients (Vespa et al., 2021). Therefore, the system of wastewater represents viruses ending up there from feces, urine, sneezing, sputum, and coughing of patients (WHO, 2020; Hokajärvi et al., 2021).

In the literature, there are some different studies about COVID-19 in wastewaters. Hart and Halden (2020) studied the computational analysis of SARS-CoV-2/COVID-19 surveillance by wastewater based epidemiology locally and globally. Orive et al. (2020) studied early SARS-CoV-2 outbreak detection by sewage-based epidemiology. Olesen et al. (2021) reviewed the lead time of wastewater based epidemiology for COVID-19. Cao and Francis (2021) studied forecasting of the community-level COVID-19 cases from SARS-CoV-2 concentration in wastewater. Mandal et al. (2021) reviewed the presence, survival, and disinfection/removal methods of coronavirus in wastewater as well as the progress of wastewater based epidemiology. Pulicharla et al. (2021) reviewed the rethinking of wastewater monitoring as a preemptive approach for COVID-19 pandemic. Cervantes-Avilés et al. (2021) reviewed the approaches applied to detect SARS-CoV-2 in wastewater and perspectives post-COVID-19.

Enteric viruses resulting from human gastrointestinal tract can exist in fecal contaminated waters. Urban wastewaters have more than 100 viral species of enteric origin, and many of them can cause diseases in humans. Viruses causing waterborne diseases are noroviruses (NoVs) and human adenoviruses (HuAdV). NoV is an unencapsulated capsid and a small single-stranded RNA genome with small round-shaped viruses with a diameter of 38-40 nm (Dias et al., 2018). NoVs are one of the most important causes of gastroenteritis outbreaks among enteric viruses (Ito et al., 2017). NoV is major cause of diarrhoeal disease in adults. NoV is known to be causing epidemic situations, particularly in slum areas with densely populated (Teunis et al., 2008; Katukiza et al., 2013; Fuhrimann et al., 2016). Sima et al. (2011) reported NoV is present in stool during acute phase of the infection and persists for 3 weeks after symptoms have subsided (Dias ve diğ., 2018). Therefore, it is not surprising to encounter NoV in domestic wastewater. Dias et al. (2018) reported occurrence of NoV in domestic wastewater. HuAdV is a medium size (with a diameter of 90-100 nm) virus with envelope-free capsid and linear double-stranded DNA genome. HuAdV is found in the stool. For this reason, it is present in raw wastewater, WWTP effluent and water environments (Dias et al., 2018). HuAdVs are the most common pathogenes associated with various clinical syndromes (e.g. conjunctivitis, gastroenteritis, and respiratory diseases (Swenson et

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al., 2003). Outbreaks of HuAdV infections occur in swimming pools, hospitals, day care centers, and military areas (Jiang, 2006). Rotavirus is one of the leading causes of childhood diarrhoea (Katukiza et al., 2013; Sigei et al., 2015; Fuhrimann et al., 2016). *Campylobacter* spp. are zoonotic bacteria causing campylobacteriosis, with *Campylobacter jejuni* (*C. jejuni*) which is common cause of diarrhoea (Kaakoush et al., 2015). Enterohemorrhagic *E. coli* (EHEC) is considered pathogenic. Serotype *E. coli* O157:H7 is responsible for largest public health impact (Okeke, 2009; Hynds et al., 2014). *Salmonella* spp. have more than two thousand sero-groups. However, *S. typhi* and *S. para-typhi* A, B and C, and the enteric salmonella strains are concern for health of human (Kariuki et al., 2015; Fuhrimann et al., 2016). Protozoan pathogens are found in water sources throughout the world (WHO, 2004). The parasitic protozoan diseases cause deaths in developing countries. Furthermore, protozoan pathogens cause important diseases in developed countries (Fletcher et al., 2012; Pulutzer and Karanis, 2016). Protozoa are unicellular eukaryotic organisms including sporozoa (e.g., *Cryptosporidium*), flagellates (e.g., *Giardia*), amoebae (e.g., *Entamoeba*) and ciliates. *Giardia* and *Cryptosporidium* are well known waterborne protozoa causing outbreaks (Karanis et al., 2007; Baldursson and Karanis, 2011; Pulutzer and Karanis, 2016) because they have high infectivity and resistance to disinfection and treatment of water (WHO, 2009; Xiao et al., 2018). In immunodeficiency human (e.g. human immunodeficiency virus infected human), the most prevalent worldwide waterborne infection producing diarrhea caused by *Cryptosporidium* and *Giardia* is more common and can threat life (Hunter and Nichols, 2002; Xiao et al., 2018). At least 8 different amoebae live in human intestinal lumen. However, they are usually accepted as commensals with exception of *E. histolytica* (Raza, 2013; Pulutzer and Karanis, 2016).

This study is the first research article study investigating SARS-CoV-2 and gastrointestinal pathogens together. This study focuses on the SARS-CoV-2 and gastrointestinal pathogens (bacterial gastroenteritis, viral gastroenteritis, enteroviruses, human parechovirus, adenoviruses, stool parasites) in a MWWTP in Turkey. The following points are aimed in our study; (i) SARS-CoV-2, which has affected the whole world, in different units of a MWWTP in Turkey was investigated. (ii) Gastrointestinal pathogens known to cause various epidemics at different times in the influent and effluent of the MWWTP were investigated. However, various studies have been done world-wide on the existence of SARS-CoV-2 in wastewater, this study is a specific study on the research on the existence of SARS-CoV-2 and gastrointestinal pathogens (Enterohemorragic/verotoxin-producing *Esherichia coli*, *Campylobacter coli/jejuni/lari* and IC, *Clostridium difficile*, *Salmonella* spp., *Shigella* spp., Enteroinvasive *Escherichia coli*, *Yersinia enterocolitica*, Noroviruses (NoroG1), Noroviruses (NoroG2), Rotaviruses (Rota), Astroviruses (Astro), Adenoviruses (HAdV), Sapoviruses (Sapo), Enteroviruses, Human Parechovirus (HPeV), Adenoviruses, *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium parvum*) in wastewater. Therefore, our study will contribute to the literature.

MATERIAL AND METHODS

Study Area and Sample Collection

In our study, municipal wastewater treatment plant was chosen as study area (Elazığ, Turkey) (Figure 1). MWWTP treats the wastewater of 383.975 people reaching the sewage treatment plant. MWWTP project flow is 820 and 1671 L/s for 2000 and 2020, respectively. MWWTP basically consists of a screen, grit removal, primary clarifier, aeration tanks and secondary clarifier, respectively. The wastewater treatment plant was revised in 2007 and operated in 2008 (Topal and Arslan Topal, 2011; Topal et al., 2014; Topal et al., 2016). The coordinates (UTM WGS84 6°) of WWTP influent and effluent points are as follows: Influent point =Y:529256, X:4271844 and effluent point= Y:529401, X:4271832. Magellan eXplorist 510 (Santa Clara, USA) was used to determine the coordinate values. Flow diagram of the MWWTP and sampling points were given in Figure 2.

Sampling points for SARS-CoV-2 analysis were carried out as follows; (1) Influent, (2) Inlet of primary clarifier unit (outlet of grid chamber unit), (3) Inlet of aeration tanks unit (outlet of primary clarifier unit), (4) Inlet of secondary clarifier unit (outlet of aeration tanks unit), (5) Effluent, and (6) Sludge thickening unit. Sampling points for gastrointestinal analysis were carried out as follows; (1) Influent and

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(2) Effluent. Composite samples from influent and effluent of MWWTP were taken. Wastewater samples for analyses of SARS-CoV-2 and gastrointestinal were collected in 0.25 L tube (sterilized before use). The samples were brought to laboratory and prepared for analysis. Gastrointestinal pathogens were examined under 4 groups. These; (i) Bacterial agents causing gastroenteritis, (ii) Viral agents causing gastroenteritis, (iii) EPA, and (iv) Stool parasites. The gastorintestinal pathogens are summarized in Table 1.

Analysis

Gastrointestinal pathogens and SARS-CoV-2 analysis were performed in the wastewater samples as follows;

The working stages of the gastrointestinal panel are as follows:

(i) Isolation stage: At this stage, DNA isolation is performed using the EZ1 device. In the gastrointestinal panel, 400 µl wastewater is taken and transferred to a 2 mL tube for wastewater isolation. This tube is placed in the appropriate place in the device. For each sample to be isolated, mix is prepared using 54.2 µl of AVE buffer, 3.8 µl of CRNA, 2 µl of internal control (IC) into separate 1.5 mL tubes. These tubes are thoroughly vortexed and spun. They are placed in the appropriate place in the device. Then, 1.5 mL tubes are placed in the section where DNA transfer will be made on the device, and EZ1 mini kit cartridges are placed in the appropriate section with pipettes and chambers. The isolation protocol is started. The isolation protocol takes 43 minutes.

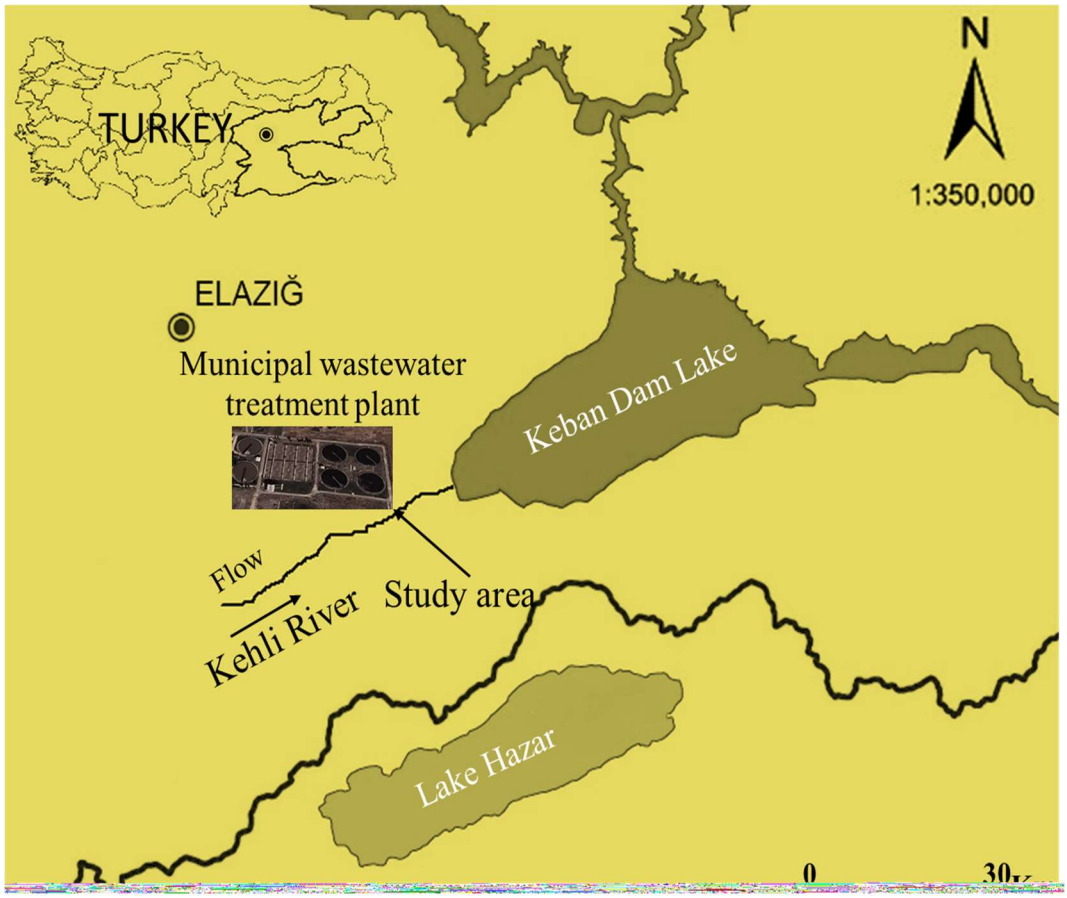

Figure 1. Study area

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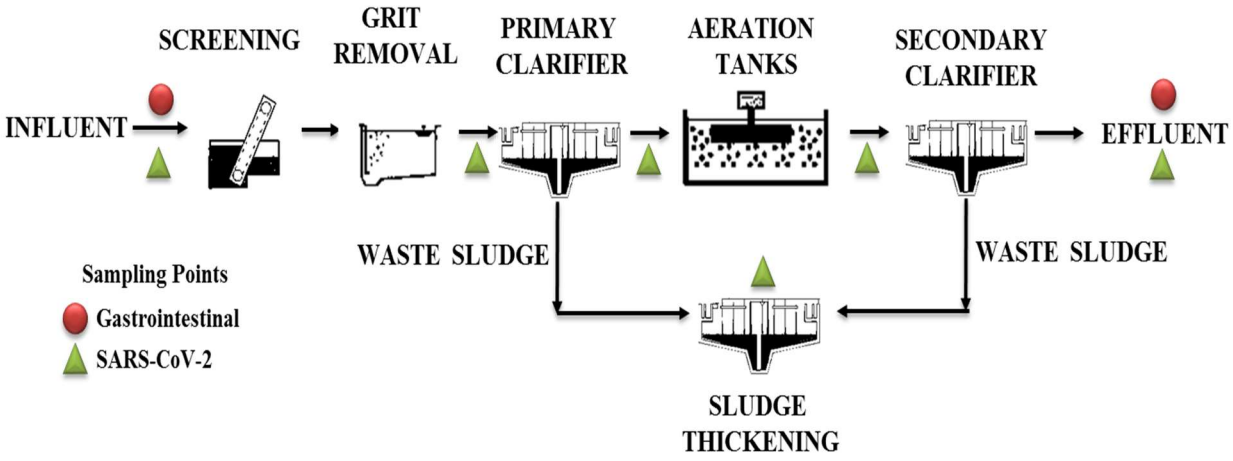

Figure 2. Flow diagram of the municipal wastewater treatment plant and sampling points

Table 1. *The gastorintestinal pathogens*

	Gastrointestinal pathogens
Bacterial gastroenteritis	Enterohemorragic/verotoxin-producing *Escherichia coli* *Campylobacter coli/jejuni/lari* and IC *Clostridium difficile* *Salmonella* spp. *Shigella* spp. Enteroinvasive *Escherichia coli* *Yersinia enterocolitica*
Viral gastroenteritis	Noroviruses (NoroG1) Noroviruses (NoroG2) Rotaviruses (Rota) Astroviruses (Astro) Adenoviruses (HAdV) Sapoviruses (Sapo)
EPA	Enteroviruses Human Parechovirus (HPeV) Adenoviruses
Stool parasites	*Entamoeba histolytica* *Giardia lamblia* *Cryptosporidium parvum*

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(ii)PCR stage: Bacterial gastroenteritis, viral gastroenteritis, EPA and fecal parasite kit boxes are removed from -20ºC. PPmix, buffer, negative control (NC), positive control (PC) are left to dissolve at room temperature. The enzyme is stored at -20ºC until the PCR mix is prepared. The buffer solute, PP mix, NC, IC, and PC are vortexed, spined. The enzyme is then removed from -20oC and spined briefly. For each sample, the mix is prepared using 12.5 µl buffer, 1.5 µl PP mix, 1 µl enzyme. This mix is distributed to the numbered 0.2 mL PCR tubes in 15 µl each. Then 10 µl. from the DNA of each sample into these 0.2 mL tubes each numbered for it is distributed. The PCR stage is started in the Fast Track Diagnostics multiplex PCR protocol in the ROTOR-GENE instrument. This protocol takes 113 min.

(iii) Analysis stage: Analysis is done through green, orange, yellow and red channels. For bacterial agents causing gastroenteritis; E. coli and Clostridium difficile (C. difficile) from green channel; Shigella and internal control from orange channel; Camplylobacter and Salmonella from red channel; Yersinia enterocolitica (Y. Enterocolitica) from yellow channel. For viral agents causing gastroenteritis; Noroviruses G2, Astroviruses, and Sapoviruses from green channel; Rotavirus and internal control from yellow channel; Norovirus G1 and Adenovirüs from red channel. For EPA; Enterovirus from green channel; Parechovirus from yellow channel; internal control from orange channel; Adenovirus from red channel. For fecal parasites; Giardia lamblia (G. lamblia) from red channel; E.histolytica from yellow channel; Cryptosporidium from orange channel. Factors located across these channels are looked at.

SARS-CoV-2 analysis was done by Reverse Transcription quantitative PCR (RT-qPCR). RT-qPCR was performed by RT-qPCR primers targeting the viral RNA-dependent RNA polymerasegene (RdRp) gene and the RT-qPCR probes SARS CoV-2 Double Gene RT-qPCR Kit (Bio-Speedy). SARS-CoV-2 Double Gene RT-qPCR kit (Bio-Speedy) is a single channel, real-time, one-step reverse transcription PCR test used for the qualitative detection of SARS-CoV-2 specific ORF1ab and N (Nucleocapsid) genes (URL, 1). The analysis of SARS-CoV-2 are briefly as follows: 400 µl were taken from the wastewater samples and loaded into the isolation device. 60 µl of RNA was obtained at the end of the isolation process. RNAs were kept in deep freeze until PCR process. PCR mixes were prepared as much as the number of samples, negative controls and positive controls. For samples and controls, a total of 140 µl of 2X Prime solution and 70 µl of Oligo mix were added to an empty, screw-capped sterile tube of 2 ml and vortex-spin was performed. 15 µl of the prepared PCR mix was distributed to 0.2 ml PCR tubes. 5 µl of the isolated RNAs were added to the same tubes by pipetting. The tubes whose caps were closed were placed in the rotor pulleys and the related COVID-19 PCR protocol was opened on the device and the PCR process was started.

RESULTS AND DISCUSSION

In our study, both gastrointestinal pathogens and SARS-CoV-2 are investigated in samples taken from different units of the MWWTP. Enterohemorragic/verotoxin-producing *E. coli, C. coli/jejuni/lari* and IC, *C. difficile*, *Salmonella* spp., *Shigella* spp., and *Yersinia enterocolitica* were determined as positive (+) in terms of bacterial agents causing gastroenteritis in samples taken from influent of MWWTP. However, Enteroinvasive *E. coli* was not observed in the influent of MWWTP. Therefore, a negative (-) result was obtained. When effluent of MWWTP were examined in terms of bacterial agents causing gastroenteritis, Enterohemorragic/verotoxin-producing *E. coli*, *C. difficile*, *Shigella* spp., *Salmonella* spp., *Y. enterocolitica* and Enteroinvasive *E.coli* were found to be negative (-).The reason why these bacterial agents causing gastroenteritis are negative in MWWTP effluent can be explained by their adherence to treatment sludge discharged from system at the wastewater treatment plant. *C. coli/jejuni/lari* and IC, which were positively (+) detected in the influent of MWWTP, were positively (+) detected in the effluent. The reason why *C. coli/jejuni/lari* and IC has a positive value in wastewater treatment plant effluent shows that treatment plant cannot eliminate these species. In addition, the absence of any disinfection system in treatment plant can explain this situation. When bacterial agents causing gastroenteritis were investigated in the effluent of MWWTP, only 1 agent out of 7 was detected as positive (+). When the influent of MWWTP were examined in terms of viral agents causing gastroenteritis, Noroviruses (NoroG1), Noroviruses (NoroG2), Rotaviruses (Rota), Astroviruses (Astro), and Adenoviruses (HAdV) were determined as positive (+).

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Sapoviruses was not found in the influent of MWWTP. Therefore, a negative (-) result was obtained. When the effluent of MWWTP were examined in terms of viral agents causing gastroenteritis, only Adenoviruses were detected as negative (-). Noroviruses (NoroG1), Noroviruses (NoroG2), Rotaviruses (Rota), and Astroviruses (Astro) were observed as positive (+). The reason why these viral gastroenteritis are positive shows that the wastewater treatment plant does not have a mechanism to remove viruses. In addition, the absence of a disinfection system of treatment plant increases possibility of viruses in effluent. When viral agents causing gastroenteritis were investigated in the effluent of MWWTP, only 4 agent out of 6 was detected as positive (+). Enteroviruses, Parechoviruses and Adenoviruses, which are other gastrointestinal pathogens and expressed as EPA, were investigated in the influent and effluent of the MWWTP. According to obtained analysis results, Enteroviruses, Parechoviruses, and Adenoviruses were found to be positive (+) in the influent of the MWWTP. When the effluent of MWWTP were examined in terms of EPA, enteroviruses was found as negative (-). When EPA was investigated in the effluent of MWWTP, only 2 agent out of 3 was detected as positive (+). When the influent of MWWTP were examined in terms of stool parasites, G. lamblia, E. histolytica, and Cryptosporidium parvum (C. parvum) were found to be positive (+). According to obtained analysis results, E. histolytica and C. parvum were determined as negative (-) in the effluent of MWWTP. When stool parsites were investigated in the effluent of MWWTP, only 1 agent out of 3 was detected as positive (+).One of the most important aims of this study was to research SARS-CoV-2 in MWWTP. For this purpose, wastewater samples were taken from every stage of MWWTP and SARS-CoV-2 analysis was performed. According to obtained analysis results, SARS-CoV-2 could not be detected in any unit of MWWTP. Hokajärvi et al. (2021) reported that persistency of non-enveloped viruses (e.g. norovirus, adenovirus, or enterovirus) is not necessarily higher than persistence of enveloped ones (e.g. SARS-CoV-2) in cold environmental conditions. In our study, we detected the occurrence of non-enveloped viruses in wastewater. Therefore, it is thought that SARS-CoV-2 can also be detected as well. The probable reasons are as follows: (i) Viruses previously investigated in researches are most commonly non-enveloped ones. However, SARS-CoV-2 is an enveloped virus. Currently, protocols for testing of SARS-CoV-2 in samples of wastewater vary a lot. Since, aim is detection of virus at low concentrations in wastewater, efficiency of procedures employed for processing samples are critical (Medema et al., 2020a). Also, developing standardized, reliable virus quantification protocols are needed (Orive et al., 2020; Hokajärvi et al., 2021). In our study, the primer used in detection of SARS-CoV-2 in samples of wastewater probably was not successful in detection.

In further studies it was aimed the changing of the primer by addressing different genes for detection of SARS-CoV-2. (ii) Hokajärvi et al. (2021) reported that SARS-CoV-2 being an enveloped virus has higher affinity for attaching onto particulate matter of wastewater in comparison with non-enveloped ones. Therefore, it can be thought that SARS-CoV-2 attached to the solids in WWTP.

CONCLUSION

In our study, bacterial and viral agents causing gastroenteritis, EPA, stool parasites, and SARS-CoV-2 in the MWWTP were investigated. In this context, important results were obtained in the study. When the effluents of MWWTP were examined in terms of gastrointestinal pathogens, *Campylobacter coli/jejuni/lari* and IC, Noroviruses (NoroG1), Noroviruses (NoroG2), Rotaviruses (Rota), Astroviruses (Astro), Parechoviruses and Adenoviruses, and *G. lamblia* were observed as positive (+). In addition, SARS-CoV-2 could not be detected in units of MWWTP. As a result, according to research findings, since pathogens are encountered in wastewater treatment plant effluents, it is necessary to have a disinfection system in the treatment plants and to monitor pathogens and SARS-CoV-2 continuously in order to protect environmental and human health.

CONFLICT OF INTEREST

The Author report no conflict of interest relevant to this article.

RESEARCH AND PUBLICATION ETHICS STATEMENT

The author declares that this study complies with research and publication ethics.

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