ORIGINAL ARTICLE

Detection of BK and JC polyomaviruses in sewage water of the urban areas of Lahore, Pakistan

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Abstract

The surveillance of sewage water has become an extremely essential tool to trace the circulation of viruses in a population and to predict the outbreak of viral diseases. Sewage monitoring is important for those viruses which cause subclinical infections since it is difficult to determine their prevalence. Polyomaviruses are ubiquitously present, circular double-stranded DNA viruses that can infect humans as well. Among all human polyomaviruses, BK polyomavirus and JC polyomavirus associated with the development of aggressive diseases in immunocompromised individuals, are highly prevalent. This study aimed to investigate the presence and the quantitative prevalence of these two disease-associated human polyomaviruses in sewage water collected from six drains of Lahore, Pakistan. The viruses present in the environmental samples were concentrated by PEG method before isolating viral nucleic acids. Conventional PCR amplifications were performed for molecular detection of BK polyomavirus and JC polyomavirus targeting their large tumor antigen genetic region. The presence of BK polyomavirus and JC polyomavirus was confirmed in the DNA extracted from concentrated sewage samples of each drain by performing both qualitative and quantitative PCR. Our data shows that the viral load ranged from 1278 to 178368 copies per μg of environmental DNA for BK polyomavirus and 5173 to 79129 copies per μg of environmental DNA for JC polyomavirus. In conclusion, here we report first time the detection of BK polyomavirus and JC polyomavirus in sewage water collected from six main drains in urban areas of Lahore, Pakistan showing the high prevalence of these viruses in the Pakistani population. This assay could be used as a proxy to determine the prevalence of these viruses in the Pakistani population.

Keywords Polyomaviruses (PyVs) · Human polyomaviruses · BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV) · Sewage and wastewater surveillance · qPCR

Introduction

Polyomaviruses (PyVs) are ubiquitously present, circular double-stranded DNA viruses whose natural hosts are primarily birds and mammals (Nomburg et al. 2022). Studies have reported that PyVs also infect the human population (Kamminga et al. 2018) The family of polyomaviruses infecting humans is rapidly expanding with 14 different species of human polyomaviruses being discovered till date (Linthorst et al. 2022). Not all of the human polyomaviruses

are associated with the development of diseases but only four human polyomaviruses are known to be associated with aggressive diseases in immunocompromised individuals (Condez et al. 2021). Of these, BK polyomaviruses (BKPyV) and JC polyomavirus (JCPyV) were the foremost human polyomaviruses to be described and to be linked with human malignancies.

BKPyV & JCPyV genomes have been found in brain cells, immune cells, tonsils, respiratory tract, blood mononuclear cells, lymph nodes, and kidneys (Ciotti et al. 2019). Both viruses cause asymptomatic primary infection at an early age and remain latent in renal tissues and B lymphocytes until the immune system is suppressed in immunocompetent individuals (Whiley et al. 2001). In immunocompromised individuals, under conditions including diabetes, organ transplantation, anti-tumor therapy, and pregnancy (Barbanti-Brodano et al. 1998; Limaye

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et al. 2001), both viruses become reactivated from latent infection to a lytic state of infection (Iaconelli et al. 2015). The reactivation of BKPyV and JCPyV is associated with the development of devastating human diseases including polyomavirus-associated nephropathy, hemorrhagic cystitis, and progressive multifocal leukoencephalopathy (Dehcheshmeh et al. 2020).

Globally, BKPyV and JCPyV are highly prevalent, infecting about 70% and more than 90% of the adult population, respectively (Bouvard et al. 2012). BKPyV and JCPyV are characterized as small non-enveloped viruses, which contain a single double-stranded DNA of approximately 5 kb enclosed in an icosahedral capsid. The genome of both viruses encompasses two transcriptional active regions separated by a hypervariable non-coding control region, harbouring an *ori* gene essential for replication and transcription of early and late regions. The early region of both polyomaviruses is involved in the expression of regulatory proteins "tumor antigens" (*T-Ag*), whereas the late region encodes capsid proteins (Van Ghelue et al. 2012). The early region of BKPyV is translated into three tumor antigens (*T-Ag*) including large tumor antigen (*LT-Ag*), small tumor antigen (*ST-Ag*), and truncated T antigen (*Trunc T-Ag*) whereas the JCPyV early region encodes some splice variants (T'135, T'136, and T'165) in addition to *LT-Ag* and *ST-Ag* (Khalili et al. 2008). The late region of BKPyV and JCPyV is translated into structural capsid proteins including viral protein 1 (VP 1), viral protein 2 (VP 2), and viral protein 3 (VP 3) as well as non-structural agnoprotein (De Gascun and Carr 2013).

Both BKPyV and JCPyV have been known to spread through the respiratory tract, blood transfusion, seminal fluid, and/or organ transplantation. However, some reports describing the shedding of these viruses through urine and feces (Condez et al. 2021), and subsequently their detection in the sewage samples suggest the possibility of transmission through the urine-oral or fecal–oral route (Dehcheshmeh et al. 2020; Condez et al. 2021). Recently, a few studies in developed countries have reported that up to 90% of the human population has been exposed to human polyomaviruses, with several infections occurring at childhood ages (Egli et al. 2009; Kean et al. 2009; Šroller et al. 2016). However, no research study has been performed about the occurrence of polyomaviruses and the potential health hazards they might be causing in the Pakistani population. Therefore, the purpose of the research project proposed here is to detect polyomaviruses in different sewage samples from the city of Lahore, by using molecular tools specifically targeting the detection of polyomaviruses. Such efforts to detect these highly important but previously neglected polyomaviruses, are important as they may yield valuable insights into the establishment of latent infections and viral carcinogenesis.

Material and methods

Collection of sewage samples and concentration of viruses

Sewage (1 L) samples were collected from different drains located in Lahore in sterile collection bottles. Adequate personal protective equipments were used by personnels collecting sewage samples. The collected sample was mixed properly before aliquoting and stored at -20 °C. To detect viruses in sewage water, viruses were concentrated by polyethylene glycol (PEG) method (Hjelmsø et al. 2017). Briefly, 25 ml of glycine buffer (0.05 M glycine, 3% beef extract, pH 9.6) was mixed with 200 ml of sewage water in a sterile beaker to release viral particles from organic materials and then subjected to centrifugation at 7000 rpm for 30 min at 4 °C. The collected supernatant was filtered through 0.45 μm polyethersulfone membrane (PES) to remove eukaryotic and bacterial cells. The pH of the filtrate was checked and adjusted to 3.7 with 6 M HCl. Both PEG 8000 (80 g/L) and NaCl (17.5 g/L) were added to the filtrate and then incubated in a shaker at 4 °C for 15–20 h. The incubated filtrate was subjected to centrifugation at 7000 rpm for 2 h to precipitate viruses. The resulting pellet was dissolved in 1 ml of phosphate buffer saline (PBS) (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4, pH 7.4) and stored at -80 °C until further processing.

DNA extraction from sewage samples

The viral genome DNA was extracted from concentrated sewage samples by the TRIzol method. Primarily, 1 ml of TRIzol reagent was mixed with 400 μl of each concentrated sample following the addition of 300 μl of chloroform in sterile eppendorf tubes. The mixture was vortexed for 10 s, incubated at room temperature for 3 min, and centrifuged at 12,000 rpm for 10 min at 4 °C. After centrifugation, the middle layer was collected into a sterile eppendorf tube, mixed with equal volume of ice chilled isopropanol, and incubated at room temperature for 10 min following centrifugation. Pellet was washed with 70% ethanol, air-dried, and dissolved in 50 μl of DEPC treated water. The quality and quantity of isolated DNA were checked by gel electrophoresis and Nanodrop 2000.

Detection of BKPyV and JCPyV in sewage water by conventional PCR

The presence of BKPyV and JCPyV were detected in sewage water by using conventional PCR. PCRs were performed using the primer pairs specifically amplifying *LT-Ag* of

BKPyV and JCPyV. A forward primer 5'-AATATTATG
CCCAGCACACATG-'3 and a reverse primer 5'-CTTTCC
CTCTGATCTACACCAG-'3 were used to amplify 171 bps
of the *Lt-Ag* region of BKPyV. Similarly, a pair of forward
primer 5'-AGAGTGTTGGGATCCTGTGTTT-'3 and reverse
primer 5'-TTGCAGGGCATTTTGTTTTTTAC-'3 were used
to amplify the 177 bps region of *LT-Ag* of JCPyV (Hussain et al. 2017). PCR was performed in a thermal cycler
(applied B® 2720 thermal cycler) using the following optimized conditions: initial denaturation of DNA at 95 °C for
5 min followed by 35 cycles at 95 °C for 30 s, 57 °C for 45 s
and 72 °C for 45 s and final extension at 72 °C for 10 min.
Genes of *LT-Ag* of BKPyV and JCPyV separately cloned in
pcDNA3 expression vectors, provided by Dr. Tommasino
Lab, International Agency for Research on Cancer (IARC)
in France, were used as a positive control for PCR reactions.
These plasmids were also used in PCR reactions to evaluate the specificity and cross-reactivity of primers for *LT-Ag* regions of BKPyV and JCPyV. The standards of known
copies of plasmids *LT*-BKPyV-pcDNA3 and *LT*-JCPyV-
pcDNA3 were used as positive controls for the preparation
of standard curves of BKPyV and JCPyV, respectively.

Preparation of positive controls for BKPyV and JCPyV

The standard curve of each virus was plotted by using a
10-fold serial dilution of the positive control plasmids i.e. LT-
BKPyV-pcDNA3 and LT-JCPyV-pcDNA3 for BKPyV and
JCPyV respectively. 10-fold serial dilution of positive control
plasmid was prepared according to this formula **Copy no of
interest = n * N$_A$** (n = mass of plasmid required for copy no/
molar mass of plasmid, N$_A$ = Avogadro's number).

Real time PCR for the quantification of BKPyV and JCPyV

Real time PCR (qRT-PCR) was performed on PikoReal real
time detection system for the detection and absolute quantification of BKPyV as well as JCPyV viral load in sewage
samples. qRT-PCR was performed using *LT-Ag* primers of
each virus. The qPCR reaction mixture (10 µl) was prepared
using 5 µl of qPCR Master Mix 2x (Thermo Fisher Scientific), 0.25 µM of each forward and reverse primer, 1 µl of
10 mg/ml bovine serum albumin (BSA), 1 µl of the template
and molecular biology grade water. The real-time PCR was
performed in triplicate for each sample, standard control
dilutions (10^{10} to 10^2 for BKPyV and 10^9 to 10^3 for JCPyV)
as well as for negative control. Amplification was performed
using initial denaturation at 95 °C for 10 min followed by
40 cycles of denaturation of 30 s at 95 °C, annealing for
30 s at 57 °C, and extension for 30 s at 72 °C with a final
extension at 72 °C for 5 min. In qRT-PCR, the melt curve

was performed at 55 °C to 95 °C. Melt curve, melt peak and
other data analysis were performed with PikoReal2.2 software. The standard amplification curve of the known quantity of plasmid DNA (LT-BKPyV-pcDNA3 and LT-JCPyV-
pcDNA3) was used to determine the unknown amount of
DNA in the sewage samples.

Results

Samples collection sites

Six main drains of the urban areas of the city of Lahore were
selected for the present study based on the declaration as
virus hotspots by the local government. These drains were
Cantonment drain (at the point of Nishat Colony), Gulshan
Ravi drain, Iqbal town drain, Mughalpura drain, Kharak
drain (at the point of Sabzazar area), and Roi (or Township)
drain. The locations of the sample collection sites are shown
in Fig. 1.

Evaluation of primers for BKPyV & JCPyV

Primer pairs targeting the *LT* regions of the BKPyV & JCPyV
were validated using the plasmids *LT*-BKPyV-pcDNA3 and
LT-JCPyV-pcDNA3, harboring the full-length *LT* regions
of the BKPyV & JCPyV, respectively. Moreover, cross-
reactivity of primers for *LT-Ag* of these viruses was evaluated among BKPyV and JCPyV. The primer set of BKPyV
and JCPyV amplified only their targets in respective viruses
showing specificity for BKPyV (Fig. 2) and JCPyV (Fig. 3).

Detection of BKPyV and JCPyV in sewage samples

The presence of BKPyV and JCPyV was detected in the
DNA extracted from concentrated sewage samples of each
drain by performing conventional PCR. The *LT*-BKPyV-
pcDNA3 and *LT*-JCPyV-pcDNA3 were used as a positive
control for BKPyV and JCPyV, respectively. The PCR
bands of size 171 bps and 177 bps confirmed the presence
of BKPyV (Fig. 4A) and JCPyV (Fig. 4B), respectively in
samples while no band appeared in the samples negative
for BKPyV and JCPyV. Our data showed that BKPyV and
JCPyV were present in all sewage water samples tested at
varying concentrations.

Real-time assay for the quantification of BKPyV and JCPyV viral loads

The qPCR amplification of the standards at tenfold serially
diluted concentrations of plasmids (*LT*-BKPyV-pcDNA3 &
LT-JCPyV-pcDNA3), was performed to prepare a calibration curve between Ct values and corresponding gene copy
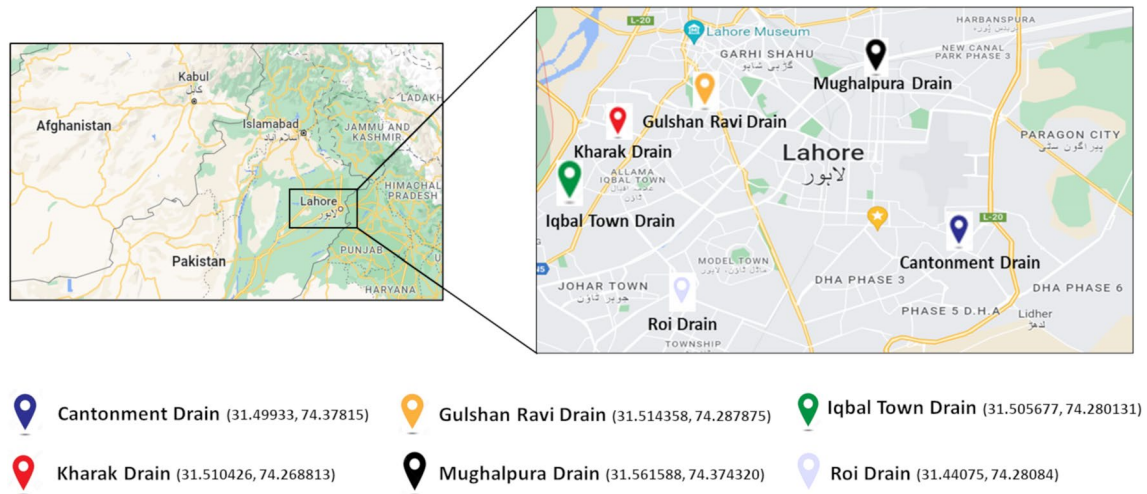
Fig. 1 Geographical illustration of sample collection sites from drains across Lahore, Pakistan

numbers for this qPCR assay. The linear range of the qPCR curve corresponded to the Ct values of 2.86 (10^{10} gene copies) to 27.58 (10^2 gene copies) and 9.41 (10^9 gene copies) to 32.64 (10^3 gene copies) for BKPyV *LT-Ag* and JCPyV *LT-Ag* primers, respectively. This curve was used for the quantification of the viral load of BKPyV and JCPyV in the sewage sample (Fig. 5).

Viral loads of BKPyV and JCPyV in sewage samples were measured by comparing their Ct value with standard control dilutions of BKPyV and JCPyV. The viral load as estimated by qPCR targeting *LT-Ag* regions of the genomes ranged from 1278 to 178368 copies per μg of environmental DNA for BKPyV and 5173 to 79129 copies per μg of environmental DNA for JCPyV.

Discussion

Amongst the known human polyomaviruses, BKPyV and JCPyV are well studied and associated with clinical diseases. BKPyV is linked with nephropathy, ureteral stenosis, and hemorrhagic cystitis whereas progressive multifocal leukoencephalopathy and various neoplasms are associated with JCPyV (Dalianis and Hirsch 2013). Both of these viruses infect humans during early childhood and establish long-lasting asymptomatic latent infection in the immunocompromised hosts. However, intense immunosuppression or immunomodulatory medication in some patients causes reactivation of the virus leading to
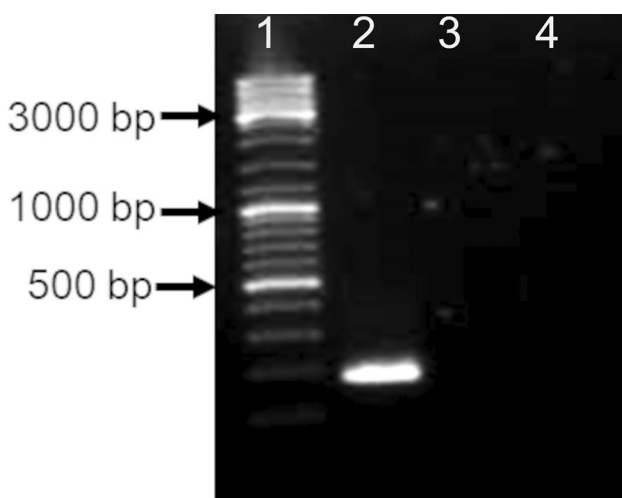

Fig. 2 Validation of BKPyV primers targeting its *LT-Ag* region. Lane 1: 1 kb ladder mix; Lane 2: BKPyV template; Lane 3: JCPyV template; Lane 4: (-) negative control
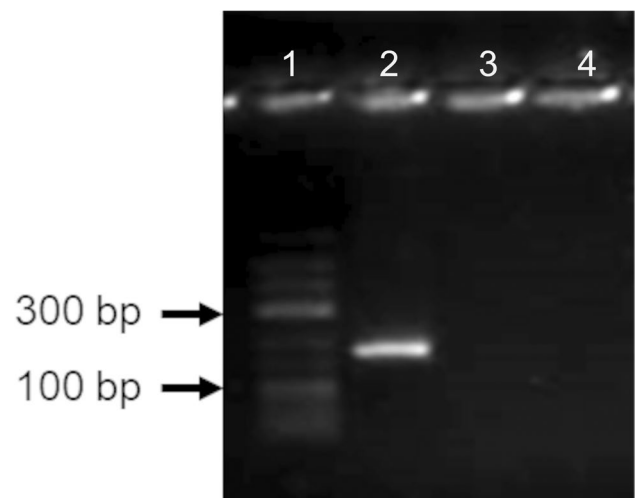

Fig. 3 Validation of JCPyV primers targeting its *LT-Ag* region. Lane 1: low range DNA ladder; Lane 2: JCPyV template; Lane 3: BKPyV template; Lane 4: (-) negative control
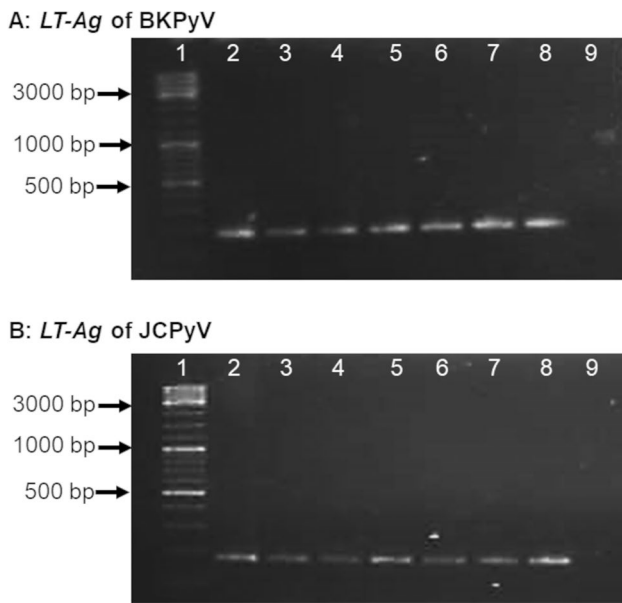
A: *LT-Ag* of BKPyV

B: *LT-Ag* of JCPyV

Fig. 4 PCR based detection of BKPyV (**A**) and JCPyV (**B**) in sewage samples targeting *LT-Ag* region. Lane 1: 1 kb ladder mix; Lane 2: (+) positive control; Lane 3: Iqbal town drain; Lane 4: Gulshan Ravi drain; Lane 5: Kharak drain; Lane 6: Roi drain; Lane 7: Cantonment drain; Lane 8: Mughalpura drain; Lane 9: (-) negative control

the cytolytic destruction of the host cells and ultimately release of the virus (Wiedinger et al. 2014). These viruses are mainly detected in the blood, cerebrospinal fluid, and urine of the infected patients. Prolonged excretion of these viruses in urine which is subsequently discharged in the wastewater causes great concern for the environment and could be a source of virus transmission. We planned this study to investigate the presence of BKPyV and JCPyV in the urban sewage of Lahore, Pakistan. Our study being the premier report on the detection of BK and JC

polyomaviruses in sewage water described the presence of these viruses in 6 different drains located in various urban areas of the city of Lahore.

Surveillance of wastewater has been proved an efficient way of tracking pathogens including viruses in communities (Hart and Halden 2020). Public health officials not only use this approach to monitor the outbreaks of diseases but also to identify threats to human health. Screening of many viruses including poliovirus, adenovirus, rotavirus, hepatitis A virus, influenza virus, noroviruses, coxsackievirus and coronavirus has been in place in many countries for decades. Very recently, wastewater-based epidemiology (WBE) has been successfully applied for the detection of the spread of SARS-CoV-2 (Farhan Ul Haque et al. 2021; Oliveira et al. 2020). The research on wastewater contamination by human polyomaviruses dates back to 2000, when the detection of BKPyV and JCPyV was first reported in urban sewage of Barcelona (Bofill-Mas et al. 2000). Later on, both of these viruses were detected in urban sewage of various geographical locations including Italy (Iaconelli et al. 2015), Brazil (Comerlato et al. 2016), Australia (Ahmed et al. 2010), and Egypt (Ahmed et al. 2019). In the present study, we reported the detection of BKPyV and JCPyV in sewage water of Lahore for the first time, indicating the ubiquitous presence of BKPyV and JCPyV in the Pakistani population. Our assay detected these viruses in all the sampling points. The possible reasons for this high detection rate could be the dense population and high prevalence of these viruses in the Pakistani population as described previously (Hussain et al. 2017). Moreover, it has been described that the detection rate of BKPyV and JCPyV varies greatly depending on the geographical regions, types of sample, and technique used.

It is worth mentioning here that certain challenges were faced during the execution of this study. The first hurdle was the concentration of viruses as water samples usually
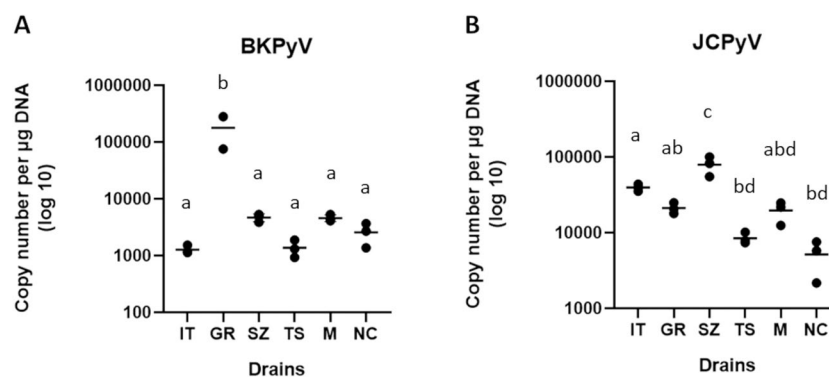

Fig. 5 Absolute quantification of *LT-Ag* copies of BKPyV (**A**) and JCPyV (**B**) by qPCR in various drains of Lahore. IT (Iqbal Town drain), GR (Gulshan Ravi drain), SZ (Kharak drain), TS (Roi drain), MP (Mughal Pura drain), and NC (Cantonment drain) represent the tested drains of Lahore. Triplicates' data in the form of dots has been presented here. Different alphabets at samples show the significant differences ($p < 0.05$) calculated by one way ANOVA and Tukey's multiple comparisons test

contain a low concentration of viruses (Girones et al. 2010). Different methods including skimmed milk flocculation, polyethylene glycol precipitation (PEG), glass wool filtration, ultrafiltration, virus adsorption, and elution have been described for virus concentration (Hoyle 1962; Hjelmsø et al. 2017; Rachmadi et al. 2016). We tried all of these methods for the concentration of the virus and found PEG the most appropriate due to its simplicity, cost-effectiveness, efficiency, and high viral recovery rate. To the best of our knowledge, the PEG method was used for the first time in this study for the concentration of BKPyV and JCPyV in sewage water samples. Extraction of nucleic acids from concentrated sewage water is necessary to detect viruses in sewage water. Various manual and kit-based methods have been reported for the extraction of nucleic acid from environmental samples (Hjelmsø et al. 2017). In this present study, two methods Exgene viral DNA/RNA kit and TRizol method were used for the extraction of nucleic acid. We found that TRizol method is better than Exgene viral DNA/RNA kit as it gave the highest yield of nucleic acids in concentrated sewage water.

Previously culture-based methods were used for the detection of viruses in water, however, these methods could only detect the infectious virus. Moreover, culture-based methods were laborious, time-consuming, and not applicable to all viruses (Haramoto et al. 2018). Recently, various molecular methods have been developed to detect viruses in water including PCR, loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA) and enzyme-linked immunosorbent assay (ELISA) (Mattison and Bidawid 2009). Of these methods, PCR is considered the most reliable method for the detection of all types of viruses in water samples (Toze 1999). Initially, in our PCR assay, we targeted the shorter fragments of *LT-Ag* and VP of both BKPyV and JCPyV. However, *LT-Ag* based PCR worked efficiently in this study. Furthermore, we used qPCR for the quantification of the BKPyV and JCPyV in the six drains samples. Our qPCR displays high sensitivity with detection limits 10^2 and 10^3 gene copy numbers for BKPyV and JCPyV, respectively.

Our data showed an overall high viral load of JCPyV in comparison to BKPyV in the samples from all drains except for Gulshan Ravi drain (GR) sample. This observation suggests the high prevalence and active infection of JCPyV in the Pakistani population in most areas. Interestingly, the shedding of JCPyV was described at a higher rate when compared with BKPyV, in the urine samples of healthy individuals in different geographical locations of the world (Boukoum et al. 2016; Urbano et al. 2016). Bofill-Mas et al. (2000) also reported that the concentration of JCPyV is higher than BKPyV in sewage water. The higher concentration of JCPyV is due to more excretion through urine not only in immunocompromised individuals but also

in immunocompetent individuals (Rachmadi et al. 2016). Moreover, Fumian et al. (2010) reported that the higher prevalence of JCPyV in sewage water is due to its higher stability in the environment. The highest viral load of JCPyV was observed in the Kharak drain (SZ) while for BKPyV the highest load was observed in Gulshan Ravi (GR) drain. Overall, the viral load in this study ranged from 1278 to 178368 copies per μg of environmental DNA for BKPyV and 5173 to 79129 copies per μg of environmental DNA for JCPyV. These results are in line with previous studies where high viral loads of JCPyV and BKPyV were described (Albinana-Gimenez et al. 2009; McQuaig et al. 2009). The difference in viral loads among these drains can be explained by the differences in population density in the surrounding regions of the drains.

Here, we reported the detection of BKPyV and JCPyV in sewage water collected from various drains in urban areas of Lahore, Pakistan showing the high prevalence of JCPyV and BKPyV in the Pakistani population. We speculate that detection of BKPyV and JCPyV in sewage water may offer a good strategy to measure the true scale of these viruses' epidemiology in the Pakistani population. However, further studies are required to develop a better understanding of the epidemiology and pathobiology of these viruses in Pakistan.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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