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Molecular Identification of Human Adenovirus Isolated from Different Wastewater Treatment Plants in Riyadh, Saudi Arabia: Surveillance and Meteorological Impacts

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Abstract: Regular water environment monitoring is crucial for minimizing contamination caused by waterborne viruses and reducing health risks. As the human adenovirus (HAdV) is linked to clinical episodes of gastroenteritis in children, the present investigation aimed to detect HAdVs in three wastewater treatment plants in Riyadh, Saudi Arabia (King Saud University (KSU-WWTP), Manfoha (MN-WWTP), and Embassy Quarter (EMB-WWTP)). The impact of seasonal variability and meteorological factors on the prevalence of HAdVs was also investigated. The HAdV hexon sequences of the isolated human adenoviruses were phylogenetically analyzed and revealed that the F species of HAdV, especially serotype 41, dominated. The highest prevalence of HAdV was detected in KSU-WWTP (83.3%), followed by MN-WWTP (75%), and EMB-WWTP (66.6%). Seasonal distribution insignificantly influenced the HAdV prevalence among sampling areas ($p > 0.05$). The highest prevalence of HAdVs (100%) was detected in late Summer and Autumn at temperatures (high: 34–43 °C, low: 18–32 °C) and moderate prevalence of 66.67% in Winter (particularly, in January and February) at lower temperature ranges (high: 26 °C, low: 10 °C–12 °C). The large variation of HAdV prevalence detected at different humidity ranges emphasized the significant impact of relative humidity on HAdV incidence in raw water of WWTPs ($p = 0.009$, $R^2 = 0.419$). In contrast, wind speed was detected to have insignificant influence on HAdV prevalence among different WWTPs ($p > 0.05$, $R^2 = 0.03$). The study provides important data for the incidence of HAdVs in wastewater treatments plants in Riyadh, Saudi Arabia, which enabled the successful management of health hazards of viral diseases transmitted via fecal-oral route. In addition, the non-significant influence of seasonal variability on HAdV prevalence highlights the potentiality of utilizing HAdVs as a potential fecal indicator of wastewater contamination.

Keywords: wastewater treatment; human adenovirus; serotype 41; seasonal variability; temperature

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1. Introduction

Enteric viruses are significant waterborne pathogens found in wastewater [1–3]. They are often isolated from feces-contaminated water and have been connected to a number of waterborne epidemics [4]. Pathogens in this category include adenoviruses, rotaviruses, hepatitis A virus, enteroviruses, and noroviruses [5]. Enteric viruses were detected from recreational beaches and groundwater for public use in the Great Lakes area, suggesting an increased public health risk from drinking or coming into contact with these waters [6,7]. Adenoviruses (HAdVs), which are abundant in wastewater, have been proposed as indicators for viral infections since they meet the majority of the requirements for an ideal indicator [8]. More than 90% of the human population is thought to be positive for one or

more adenovirus serotypes [9]. Human adenoviruses are more prevalent in sewage than other enteric viruses and are discharged in high proportions by infected people (up to 10^{11} viral particles/gram of feces) [10]. Transmission mechanisms of adenoviruses include the fecal-oral pathway and inhalation of aerosols [11]. Adenoviruses have been linked with epidemics in numerous contexts, including hospitals [12], day care facilities [13], swimming pools [14], and schools [15]. Furthermore, adenoviruses are one of the main causes of clinical disorders such as gastroenteritis, hemorrhagic cystitis, conjunctivitis, respiratory ailments, and systemic infections [16]. The six genera of the Adenoviridae family that have been classified to date are mastadenovirus, ichtadenovirus, atadenovirus, siadenovirus, aviadenovirus, and testadenovirus [17]. Human adenoviruses are classified into seven species (A–G) and more than 110 types within the Adenoviridae family [18]. Pathogenesis is caused by adenovirus infections in a range of human organs. Adenoviruses B and C are the most prevalent respiratory infections, with Adenoviruses species A affecting the respiratory system in immunodeficient individuals, and Adenoviruses F, including serotypes 40 and 41, recognized as one of the main viruses causing infantile gastroenteritis [19]. Human adenovirus is an enteric, non-enveloped, and icosahedral particle with a double-stranded, linear DNA genome of 34–36 kb [20]. They are now classified into seven HAdV species, A-G, as well as newly found adenovirus types that are constantly appearing [21]. Adenoviruses, like the majority of enteric viruses, are more resilient to environmental conditions and even sewage treatment techniques than the fecal indicator bacteria now in use [20]. Moreover, direct sequencing of PCR products, as well as sequence analysis of cloned PCR products, have been routinely employed to study adenoviruses in aquatic settings [22,23]. The research regarding the molecular detection of HAdVs in Saudi Arabia is scanty; hence, the aim of the current investigation was to detect the prevalence of HAdVs in wastewater samples collected from wastewater treatment plants at three different locations in Riyadh, Saudi Arabia. Furthermore, the impact of environmental conditions on the prevalence of HAdVs was also detected. Finally, the sequences of the detected HAdVs were phylogenetically analyzed to detect their correlation with reference strains in GenBank.

2. Materials and Methods

2.1. Sample Collection

From January 2022 to December 2022, 36 untreated wastewater samples were collected monthly at a rate of three samples per month from the inlets of the three different wastewater treatment plants (WWTPs) in Riyadh, Saudi Arabia. The sampling locations were King Saud University wastewater treatment plants (KSU-WWTP), Manfoha wastewater treatment plants (MN-WWTP), and Embassy Quarter wastewater treatment plants (EMB-WWTP) in Riyadh, Saudi Arabia. The latitude and longitude of the sampling locations are shown in Figure 1. In sterile 200-mL plastic bottles, samples were collected and transferred to the laboratory on dry ice. The data for temperature, relative humidity, and wind speed were collected on each sample day in order to study the influence of the weather on viral persistence.

2.2. Viral Concentration

Adenovirus (Adv) was concentrated using the polyethylene glycol (PEG) precipitation process [24]. Briefly, 200 mL of wastewater was combined with 25 mL of glycine buffer (0.05 M glycine and 0.3 g/L beef extract) to detach virions bound to the organic material, and the pH was adjusted to 9.6 with 1 M NaOH before centrifugation at 8000× *g* for 30 min. Afterwards, the supernatant was withdrawn using sterile syringe then filtered using Millipore filter (0.22 µm) for the removal of bacterial cell and the other unfavorable debris. The filtrate was then treated with PEG 8000 (80 g/L) and sodium chloride (17.5 g/L) followed by stirring by a magnetic stirrer overnight (100 rpm) at room temperature for viral precipitation. Centrifugation of the filtrate was then performed at 13,000× *g* for harvesting

viral particles then the pellet was eluted in 1 mL phosphate buffer saline (PBS) and finally stored at −80 °C for further experimentations.

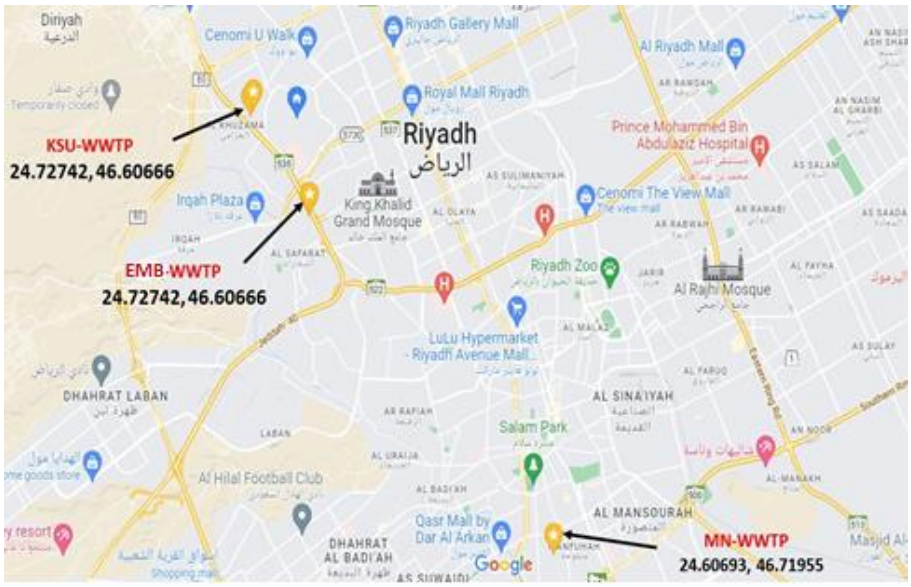

Figure 1. Latitude and longitude of sampling locations in Riyadh, Saudi Arabia.

2.3. DNA Extraction and Specific PCR Detection

Extraction of genomic DNA of HAdVs was conducted for the concentrated 36 wastewater samples using a DNeasy PowerWater Kit (Qiagen GmbH, Hilden, Germany). The viral DNA of Human adenoviruses (HAdV) was directly detected in a 20 µL reaction mixture composed of 2 µL DNA template, 300 nM AdFhex-F: 5′-GCCACCGATACCTACTTCAGCCTG-3′ as forward primer and 300 nM AdFhex-R: 5′-GGCAGTGCCGGAGTAGGGTTTAAA-3′ as a reverse primer targeting hexon gene and 1 X Phusion Master Mix under the following reaction conditions: 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s and 72 °C for 30 s each, and final extension at 72 °C for 5 min. Positive control was acquired for HAdV virus which was obtained from the Virology Unit, King Khalid University, Hospital, Riyadh, Saudi Arabia.

2.4. Amplicon Purification and Sequencing

The 261-bp amplicons for HAdV were excised and purified using the Wizard SV Gel and PCR Clean-Up System (Promega Co., Madison, WI, USA), in accordance with the manufacturer's instructions. Afterwards, the attained amplicons were then sequenced using the ABI genetic analyzer 3130Xl (Applied Biosystems®, Carlsbad, CA, USA).

2.5. Phylogenetic Analysis

The identified HAdV nucleotide sequences were examined using the MEGA X program and compared to BLAST sequences. ClustalW was used to create the sequence alignments. The phylogenetic tree was built using the minimal Bayesian information criteria and the best-fit model of nucleotide substitution. The bootstrapping of 1000 replicates was used to evaluate the reliability of the phylogenetic tree. The Kimura three-parameter approach was used to calculate genetic distances according to the best fitting substitution model (Table S1).

2.6. Statistical Analysis

Pearson's correlation coefficient matrix was used to investigate possible correlations between the various sample locations during a one-year period. To investigate the relevance of the influence of meteorological parameters (including temperature, wind speed, and relative humidity (RH%)) on HAdV prevalence independent of sample region, a one-way

analysis of variance was performed. Linear curve fitting was used to match the connections between distinct sample sites, as dependent variables, and climatic conditions, as independent variables. The XL-STAT statistics package software was used for all statistical studies (Ver. 2019, Excel Add-ins soft SARL, New York, NY, USA).

3. Results

3.1. Prevalence of HAdVs in Sampling Areas

Out of 36 wastewater samples tested for HAdVs, 27 (75%) were found to be positive, as denoted by the detection of the 261-bp amplicon (Figure 2). The highest HAdV prevalence was detected in KSU-WWTP (83.3%), followed by MN-WWTP (75%), and EMB-WWTP (66.6%) (Table 1). The molecular characterization of amplicon sequences by Sanger sequencing showed the presence of 20 sequences; however, seven sequences were not characterized owing to overlapped electropherograms. Moreover, these obtained sequences underwent phylogenetic analysis to define the possible serotype, detect any sequence variation, and to check any possible imported strains.

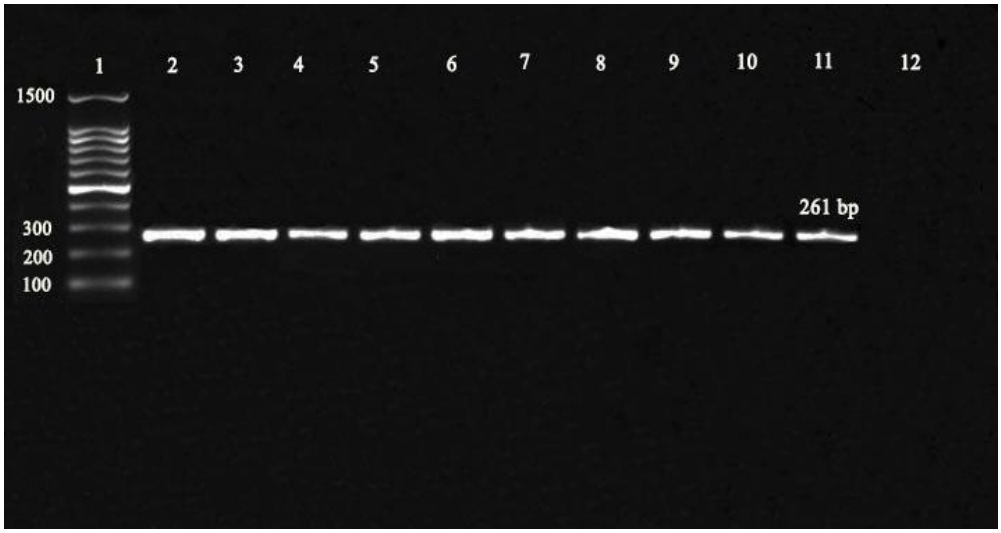

Figure 2. Gel image of the PCR product of HAdV. Lane 1, DNA marker (100–1500 bp); lane 12, negative control; lane 11, positive control; lanes 2 to 10, 261-bp HAdV ampilcons.

Table 1. HAdV prevalence in different water sample locations.

Sampling Area	HAdVs +ve	HAdV Prevalence %
EMB-WWTP	8	66.6%
KSU-WWTP	10	83.3%
MN-WWTP	9	75%

3.2. Predominance of HAdV Serotype 41

The phylogenetic tree illustrated the typical relationship of the HAdV hexon sequences obtained from all sites with serotype 41 followed by serotype 40 of HAdV (type F) (Figure 3). Pairwise distancing analysis uncovered five different HAdV isolates, and most of them (4/5) were collected from KSU-WWTP (Table S2). Brazilian HAdV isolates were the closest to our recovered sequences from the entire locations. However, HAdV recovered sequences from KSU-WTTP, in particular K19-2B64 and K20-2B64, showed the closest relationship with multiple Brazilian isolates as each showed a delicate clustering with specific Brazilian sequences. For instance, K19-2B64 was closely related to five Brazilian HAdV sequences including AD5764, AD6026, AD4578, AD01-71218, and AD102 (d = 0.0000, Table S3) than HAdV sequences isolated from irrigation water in Riyadh, Saudi Arabia (d = 0.0038467). Interestingly, K20-2B64 shared the same sequence identity as the HAdV

sequence (IW isolate) previously recovered from irrigation water in the same area of KSU-WWTP (d = 0.0000, Table S3) rather than that from the landfill wastewater or Wadi Hanifa in Riyadh (d = 0.00384). Since the Brazilian sequences, similar to our sequences, were recovered at different time periods, the time-dependent molecular divergence was taken into account and was displayed in the timetree denoted by Figure S1. The timetree showed that K23-2B64, K26-2B64, and 10UA-2B64 could have earlier molecular divergence than the other sequences that could be the origin of the other sequences and provide interpretation of the distant relationship to sequences previously recovered from the same region or from even the Brazilian sequences. On the contrary, K19-2B64 and K20-2B64 showed later molecular divergence in 2007 and 2003, respectively and consequently a closer relationship to these Brazilian sequences in each cluster. However, the large node height error bar could indicate that the molecular divergence could even have occurred earlier.

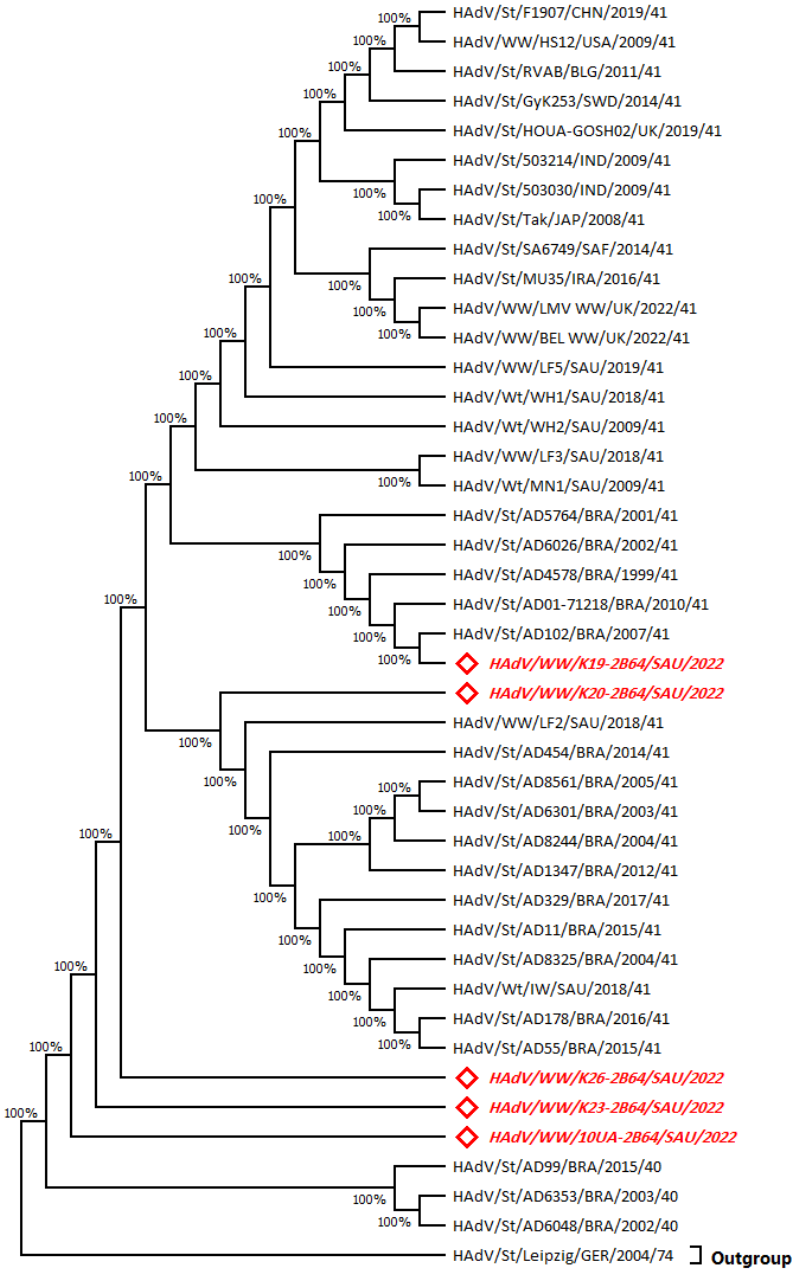

Figure 3. Phylogenetic tree for the HAdV hexon-derived sequences constructed by the maximum likelihood method and Tamura three-parameter model.

The evolutionary history was inferred by using the maximum likelihood method and Tamura 3-parameter model. The tree with the highest log likelihood (−742.94) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with a superior log likelihood value. The proportion of sites where at least one unambiguous base is present in at least one sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 43 nucleotide sequences. The rate variation model was permitted to be evolutionarily uniform for several sites, according to the best fitting substitution model validation (Table S1). The horizontal distance connecting two HAdV sequences is proportional to the genetic distance between these two HAdV sequences. The distance is expressed as the number of nucleotide substitutions per site. HAdV serotype 74 was used as an outgroup. Accession numbers of sequences used for phylogenetic analysis are displayed in Table S4. The red italicized sequences denote the current study sequences.

3.3. Seasonal Distribution of HAdV

HAdV showed various distributions over the different seasons. Overall, the highest HAdV prevalence of 22.22% was observed in Autumn season. However, HAdV recorded the least prevalence (11.11%) in Spring. In the same manner, the highest HAdV prevalence (100%) was recorded in all sampling locations in the Autumn season (Figure 4). Seasonal distribution insignificantly influenced the HAdV prevalence in all sampling areas ($p > 0.05$).

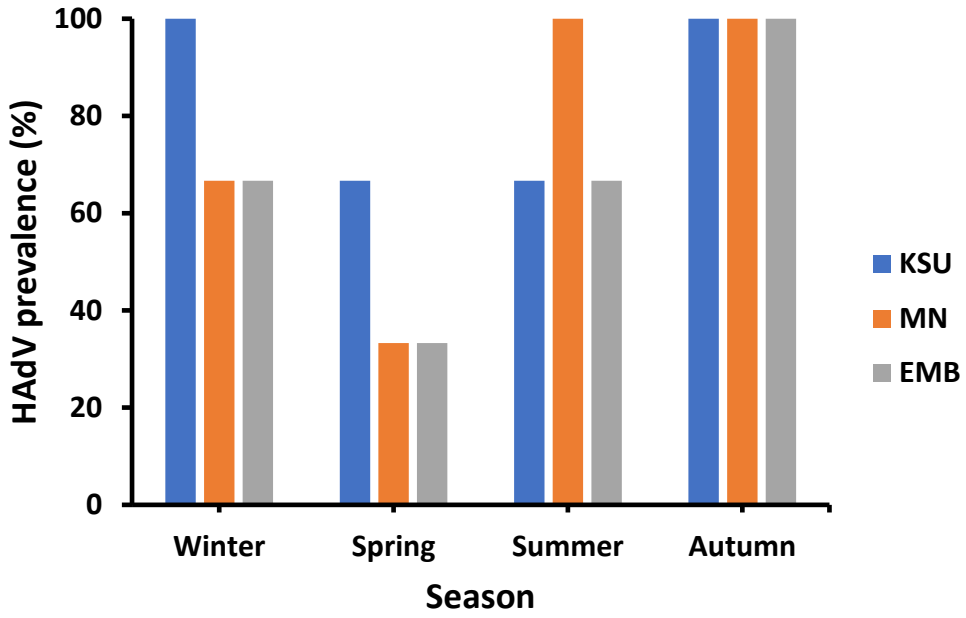

Figure 4. Seasonal prevalence of HAdVs among sampling areas.

3.4. Effect of Environmental Conditions on HAdV Prevalence

3.4.1. Temperature Variation Influence on HAdV Incidence

The lowest prevalence (33.33%) generally occurred in Spring and early Summer at temperatures (high: 30–44 °C, low: 20–30 °C), particularly during March to June (Figure 5). Notably, HAdV prevalence displayed a different pattern with the highest prevalence of 100% in late Summer and Autumn at temperatures (high: 34–43 °C, low: 18–32 °C) and moderate prevalence of 66.67% in Winter (particularly, in January and February) at lower temperature ranges (high: 26 °C, low: 10–12 °C). Consequently, the high temperature ranges were found to have a potentially significant influence on the prevalence of HAdV ($p = 0.001$, $R^2 = 0.689$; Table 2). On the other hand, the HAdV prevalence was insignificantly influenced by low temperature ranges ($p > 0.05$). Likewise, the segregation of sampling areas depicted lack of significant impact of low or high temperature on prevalence of

HAdV ($p > 0.05$). However, the highest HAdV prevalence was mostly favored in at ≥28 °C in MN-WWTP in the low temperature range, which is equivalent to ≥41 °C in the high temperature range (Figure 6). Surprisingly, the HAdV prevalence detected at EMB-WWTP was the highest at lower temperature ranges (low: 16–21 °C, high: 26–30 °C).

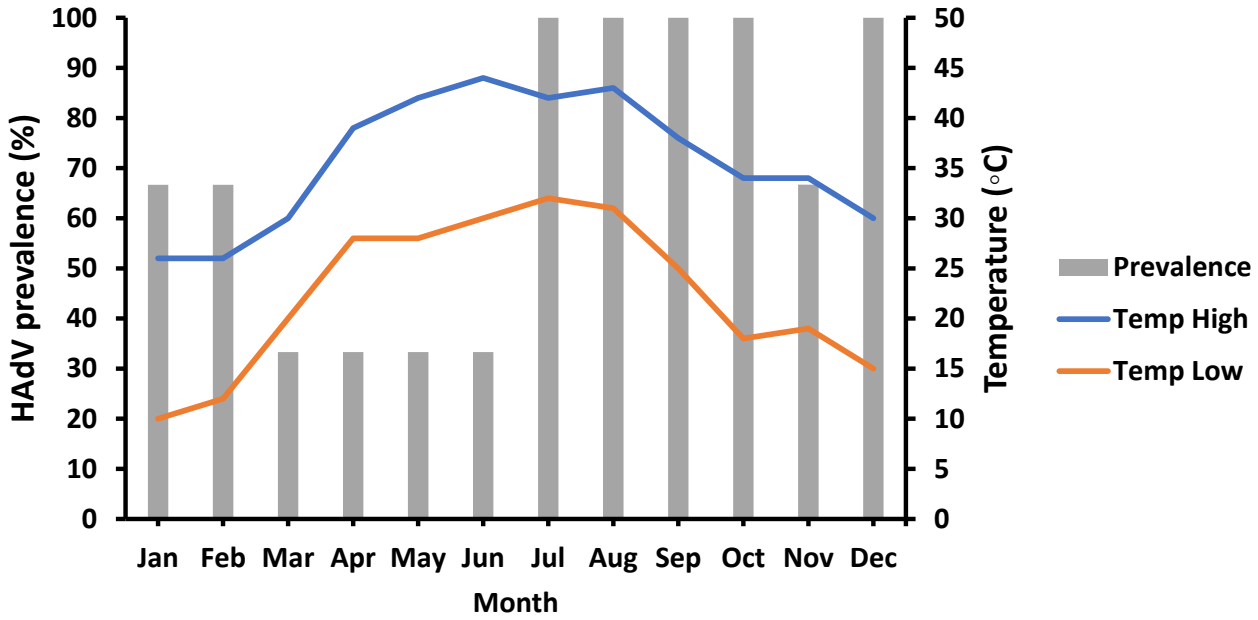

Figure 5. Temperature variation influence on the HAdV prevalence in wastewater samples. "Temp Low" refers to the average low temperature and "Temp High" refers to the average high temperature.

Table 2. Impact significance of environmental factors on the HAdV prevalence.

Environmental Factor	R^2	RMSE	Equation
High temperature (T_H)	**0.689** *	4.575	$Prev_{HAdV} = -6.15 + 0.79 \times T_H$
Low temperature (T_L)	0	4.571	$Prev_{HAdV} = 18.055556$
Relative humidity (RH%)	**0.419** **	8.439	$Prev_{HAdV} = 25.926 - 0.525 \times RH\%$
Wind speed (WS)	0.03	17.413	$Prev_{HAdV} = 16.0067 + 0.358 \times WS$

Notes: *Prev* denotes the prevalence of virus. RMSE denotes the root mean squared error. *: significant at $p = 0.001$, **: significant at $p = 0.009$.

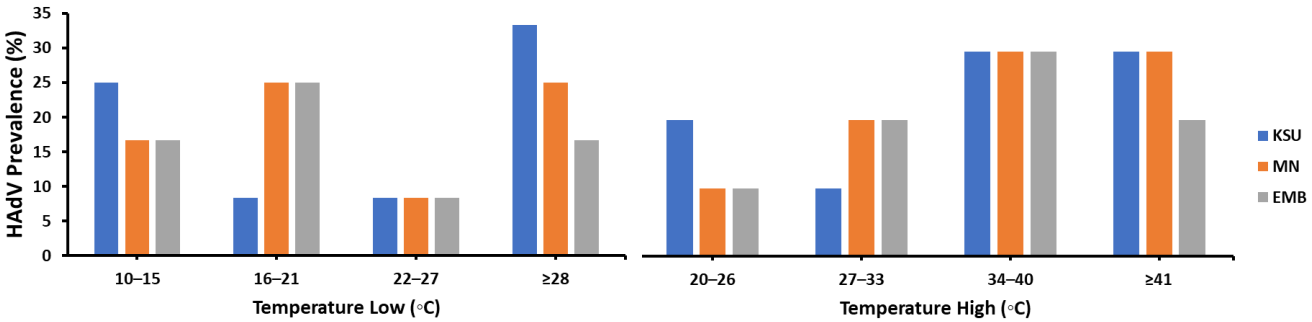

Figure 6. Temperature variation impact on HAdV prevalence in different sampling areas.

3.4.2. Humidity Variation Influenced HAdV Incidence

HAdV showed various distributions among the relative humidity ranges, favoring the lowest humidity range (6–14%) at all sampling locations with a prevalence of 33.3% (Figure 7). However, no HAdV prevalence (0%) was detected at humidity ranges of 24–32% and 33–41%, at KSU-/EMB-WWTP and MN-/EMB-WWTP, respectively. The large variation

in HAdV prevalence observed at different humidity ranges highlighted the significant influence of relative humidity on HAdV occurrence in raw water of WWTPs (p = 0.009, R^2 = 0.419; Table 2).

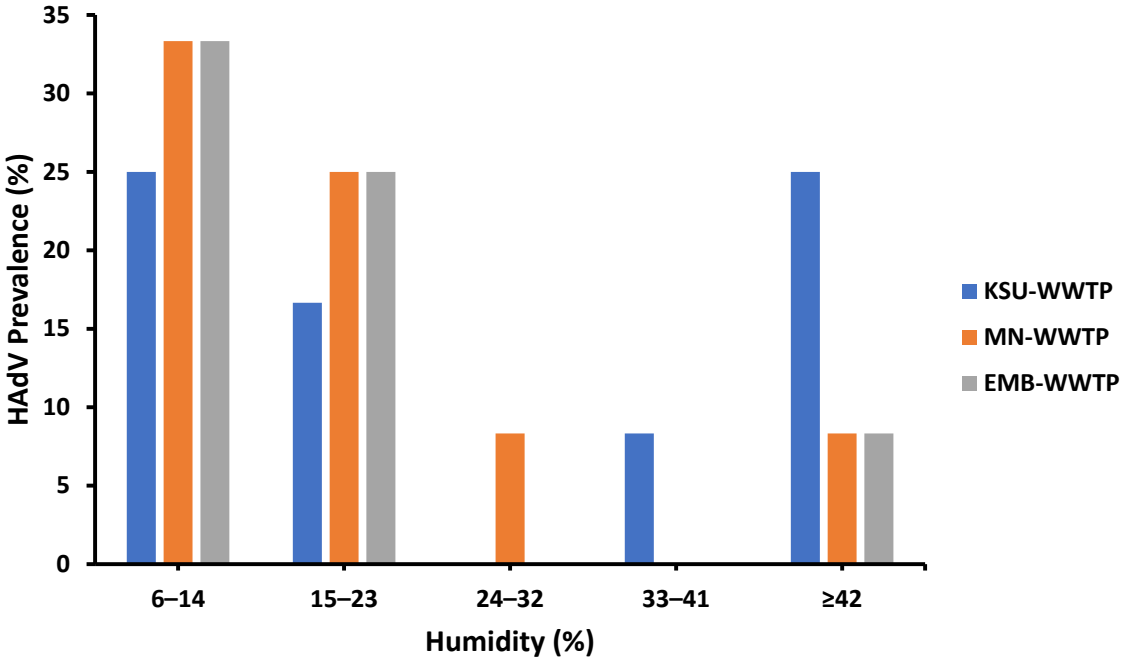

Figure 7. Impact of humidity variation on the HAdV prevalence (%) in different sampling areas.

3.4.3. Wind Speed Influence on HAdV Incidence

HAdV showed a different occurrence frequency at the various ranges of wind speed. The highest HAdV prevalence was detected at a relatively high wind speed range (15–21 km/h). Whereas the wind speed ranges of 8–14 km/h and ≥22 km/h were associated with no HAdV incidence in raw water of KSU-/EMB-WWTP and KSU-WWTP, respectively (Figure 8). Despite the HAdV occurrence pattern, wind speed was found to have insignificant influence on HAdV prevalence in the entire WWTPs ($p > 0.05$, R^2 = 0.03, Table 2).

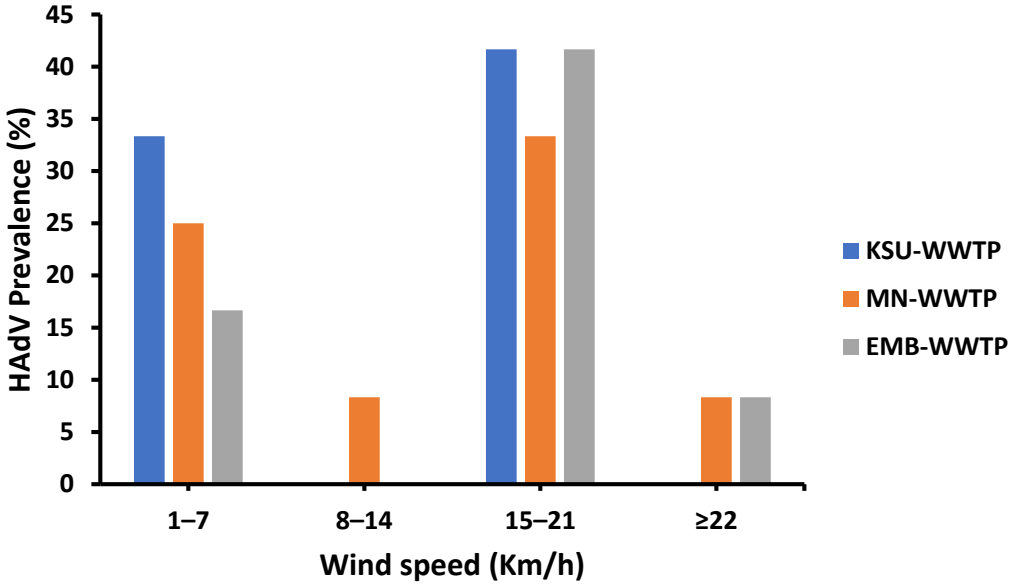

Figure 8. Impact of wind speed ranges (km/h) on the HAdV prevalence (%) in different sampling areas.

4. Discussion

Human adenoviruses are considered important indicators of fecal contamination owing to their wide detection in all types of water throughout the year and also due to their ability to resist the sewage treatment process [17]. Moreover, HAdVs are a major cause of clinical infections, including gastroenteritis, ocular, respiratory diseases, conjunctivitis, hemorrhagic cystitis, and chronic systemic infections in immunocompromised patients [25]. The continuous surveillance of human adenoviruses is of significant importance because these viruses account for 2–10% of diarrheal cases [26]. Hence, the current investigation focused on the detection of HAdVs in wastewater treatment plants in three different locations at Riyadh, Saudi Arabia. The highest prevalence of HAdVs was detected in KSU-WWTP, recording a relative percentage of 83.3%, followed by MN-WWTP (75%), and EMB-WWTP (66.6%). Our results were in accordance with those of a previous study, which reported the detection of HAdVs in 84.4% of raw wastewater samples collected from the Zenin wastewater treatment plant (WWTP), followed by treated sewage (50%) [22]. The HAdV prevalence in Manfoha wastewater treatment was higher than that detected in a prior study at the same location, which emphasizes the importance of continuous surveillance of HAdVs in wastewater samples as these viruses are correlated with gastroenteritis cases among children in hospitals [18].

The various species of adenoviruses are found in distinct tissue tropism, resulting in equally diverse clinical symptoms [27–29]. In this setting, species F and G (serotypes 40 and 41) affect the gastrointestinal tract whereas, species B, C, and E (serotypes 3, 5, and 7) primarily cause respiratory infections [30]. The current study revealed that the HAdVs belong to species F which was previously reported to be associated with gastroenteritis in children [31,32]. Phylogenetic analysis revealed that the isolated HAdV cluster with serotype 41, which had previously been found in children suffering from gastroenteritis in Riyadh, Jeddah, and Mecca in Saudi Arabia [33].

A previous study confirmed that the AdV prevalence was largely stable throughout the year, showing no seasonal effect on the removal of these viruses in wastewater treatment [13]. This finding was consistent with that study's assertion that the seasonal variability insignificantly influenced the HAdV prevalence in the locations under investigation ($p > 0.05$). The occurrence of HAdV in sewage with little or insignificant seasonal variability supported the hypothesis mentioned elsewhere that HAdV occurrence in the aquatic environment is most likely owing to potential contamination with untreated or poorly treated human sewage [34]. Moreover, the lack of seasonal impact on HAdV prevalence implicates the potentiality of HAdV as a quality indicator of water resources [35,36]. However, the highest HAdV prevalence of 22.22% was observed in the Autumn season while the lowest prevalence was detected in Spring (11.11%). This finding was in conflict with that of previous investigations conducted in China, Korea, and Egypt, which found that the highest prevalence of HAdVs was detected in Summer rather than Autumn [22,37,38]. Interestingly, HAdV prevalence was 100% in late Summer and Autumn and of moderate prevalence in Winter, with the lowest prevalence recorded in Spring and early Summer, particularly during March to June. However, a recent research found that HAdVs were more often detected on days with low temperatures (20 °C), mostly during the Winter and Spring seasons [39]. The discrepancy of our results with previous findings could be owing to geographical and temporal considerations. The HAdV prevalence in our study was insignificantly influenced by low temperature ranges, which implicates the stability of HAdV with no favorable distribution pattern even at low temperatures, which agrees a previous study that recorded twice more HAdV prevalence during high temperature seasons [37]; similar to the higher HAdV prevalence recorded in China [38]. On the contrary, a study conducted in Saudi Arabia, reported the highest HAdV prevalence at 5/6 locations favoring lower temperature ranges (22–25 °C) compared to our study [18]. The difference could be owing to the sample source since the later study was concerned with treated water, irrigation water, and surface water (lakes) as well as the temporal variation. Moreover, meteorological factors including temperature, wind speed, and humidity were

reported to have no significant impact on HAdV prevalence with few exceptions [37]. However, the HAdV distribution varied across relative humidity ranges, preferring the lowest humidity range (6–14%) at all sample sites, with a prevalence of 33.3%. Nevertheless, no HAdV prevalence (0%) was identified in humidity ranges of 24–32% and 33–41%, at KSU-/EMB-WWTP and MN-/EMB-WWTP, respectively. This finding was contrasted with that of a previous report which demonstrated that humidity had no influence on HAdV prevalence [40]. The highest HAdV prevalence was identified at rather high wind speeds (15–21 km/h); however, wind speed was shown to have an insignificant influence on HAdV prevalence over the investigated wastewater treatment plants, and this coincides with the prior report, which indicated that the virus was detected in low concentrations at extremely low wind speeds [41].

5. Conclusions

The present investigation found that species F, serotype 41 predominated among the identified HAdV strains in various wastewater treatment facilities. Since this serotype has been connected to gastroenteritis in children, ongoing monitoring of HAdVs gives novel data that are critical for fecal contamination management. The research confirmed that HAdVs were unaffected by seasonal variation and were abundant in wastewater samples, suggesting their potential use as a fecal pollution indicator.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15071367/s1, Figure S1. Timetree for HAdV evolutionary analysis by Maximum Likelihood method; Table S1. Maximum Likelihood fits of 24 different nucleotide substitution models; Table S2. Estimates of evolutionary divergence between HAdV sequences recovered from WWTPs raw water in Riyadh, Saudi Arabia; Table S3. Evolutionary divergence estimates between human Adenovirus sequences; Table S4. Full description of sequences used for phylogenetic analysis of human adenovirus isolates.

Author Contributions: Conceptualization, I.N., A.H. and S.E.; methodology, K.M., I.N. and I.A.-A.; validation, K.M., I.N. and A.A.; formal analysis, K.M., I.N., I.A.-A. and M.T.Y.; investigation, K.M. and I.N.; resources, S.E.; data curation, I.N.; writing—original draft preparation, K.M., I.N. and M.T.Y.; writing—review and editing, K.M., I.N., A.H., M.T.Y., A.A., I.A.-A. and S.E.; supervision, I.N., A.H. and S.E.; funding acquisition, S.E. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The sequences used in this study for phylogenetic analysis are openly available in NCBI GenBank repository with accession numbers mentioned in Table S4.

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Conflicts of Interest: The authors declare no conflict of interest.

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