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Removal of human astroviruses from hospital wastewater by two biological treatment methods: natural oxidizing lagoons and rotating biodisks

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ABSTRACT

Rotating biological disks and natural oxidizing lagoons are processes used to treat wastewater. These procedures were used in the present work and were situated in a pilot wastewater treatment plant situated in the residential area of Tunis City in Tunisia and received hospital wastewater from three nearby different clinics. This wastewater was very rich in various types of pathogens. Consequently, the monitoring of this treatment plant for hospital wastewater can be an appropriate approach to the study of the circulation of enteric viruses, such as human astroviruses. Wastewater samples (n = 102) were collected at the exit of the five basins from natural oxidizing lagoons and at the outlet of the rotating biodisks. Human astroviruses were identified in 55% (n = 56) of the wastewater samples by reverse transcription polymerase chain reaction (RT-PCR). An important increase was found in the frequencies of human astroviruses from the first to last lagoons of the natural oxidizing lagoon and at the exit of the rotating biological disks. Thus, these results showed the ineffectiveness of the two biological treatment methods studied for the human astroviruses removal. In addition, the data of this investigation pointed out the first detection of human astroviruses (genotype 1 and 6) in wastewater samples in Tunisia. These findings highlighted the inadequacy of the sanitary quality of treated wastewater for recycling, agricultural reuse and discharge into the receiving waters.

Keywords: Human astroviruses; Hospital wastewater; Rotating biological disks; Natural oxidizing lagoon; Removal

1. Introduction

Waterborne infections have been proven to be important in outbreaks of gastroenteritis throughout the world [1]. Despite the progressive application of improved sanitary conditions, faecal contamination remains an emerging problem. Diarrhoea remains the second leading cause of death among kids under 5 years, making nearly 1.3 million preventable deaths each year, primarily in developing rural areas [2]. Enteric viruses are usually present in environmental water and represent the major cause of waterborne infections and outbreaks [3]. Human astroviruses (HAstVs) are a diarrhoea-causing. It also cause other symptoms of acute

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gastroenteritis in children and adults in all areas of the globe, including Tunisia [4,5]. Waterborne human astroviruses, originating from polluted groundwater, surface water, sewerage, water, river water, open water and drinking water, is a serious and significant public health concern [6,7]. Once excreted in faeces at high concentrations, these viruses are present in wastewater where they are only partially removed by treatment processes. They show resistance against different physical and chemical agents, they are able to maintain their infectivity at 60°C for 10 min and to resist to treatment at pH 3 [8]. The resistance of these viruses to the treatment process facilitates their diffusion and their transmission by the faecal–oral route [3].

Human astroviruses are non-enveloped and positive-sense single-stranded RNA viruses containing a 6.8 kb poly-adenylated genome [9]. These viruses belong to the genus *Mamastovirus*, which is composed of various species (Mamastrovirus 1–19). Human astroviruses belong to the *Astroviridae* family within the *Mamastrovirus* genus [9]. Moreover, *Mamastrovirus* 1 comprises eight genotypes (HAstV-1 to HAstV-8), being HAstV-1, HAstV-2, HAstV-4 and HAstV-8 subdivided into 6, 4, 3 and 3 lineages, respectively [10]. Of these human astroviruses, HAstV-1 was recognized as the most frequent genotype in Tunisia and in the world [4,5,10–12]. In Tunisia, the identification of human astroviruses is well documented in clinical [5,13] and in shellfish samples [14]. Nevertheless, this virus was not reported in the first Tunisian environmental study on wastewater samples realized by Sdiri-Loulizi et al. [15].

The main goals of the present study were (i) the molecular detection of human astroviruses in hospital wastewater in Tunisia; (ii) the assessment of the efficiency of two secondary biological treatments: natural oxidizing lagoon or ponds and rotating biological disks (biodisks), for human astroviruses removal.

2. Materials and methods

2.1. Study area

The wastewater treatment system of the pilot wastewater treatment plant d'El-Menzeh I in Tunis City treats domestic and hospital wastewater. This pilot plant consists of four wastewater treatment lines: the line of biological rotating disks or biodisks, the line of natural oxidation lagoon or pond formed by five different lagoon basins (B_1–B_5), the line of massif underground spreading filter, and the line of trickling filters [16–18]. The location site of pilot wastewater treatment plant and the simple schematic of the two biological treatment procedures were depicted in Fig. 1. In addition, the main physical and geometric characteristics of the two biological wastewater treatment procedures studied: natural oxidizing lagoons or ponds and rotating biodisks were defined in the previous work of Ibrahim et al. [18].

During the year of study, at different points of the two treatment procedures, wastewater samples were collected. A total of 102 of wastewater samples were taken from the two studied treatment procedures. Sampling was carried out every month, there is at least one sample of wastewater during all the months of the year 2011. The exception of the August month where no samples were taken for a practical reason of pilot wastewater treatment plant staff availability. Thus, 1 L of wastewater was sampled at the outlet of the rotating biological disks line (D) and of each lagoon basin (B_1, B_2, B_3, B_4 and B_5) from natural oxidizing lagoons at different frequency rate.

2.2. Astrovirus molecular detection

2.2.1. Viruses' extraction

The detection of viral particles in the wastewater samples was carried out in accordance with the protocol of the

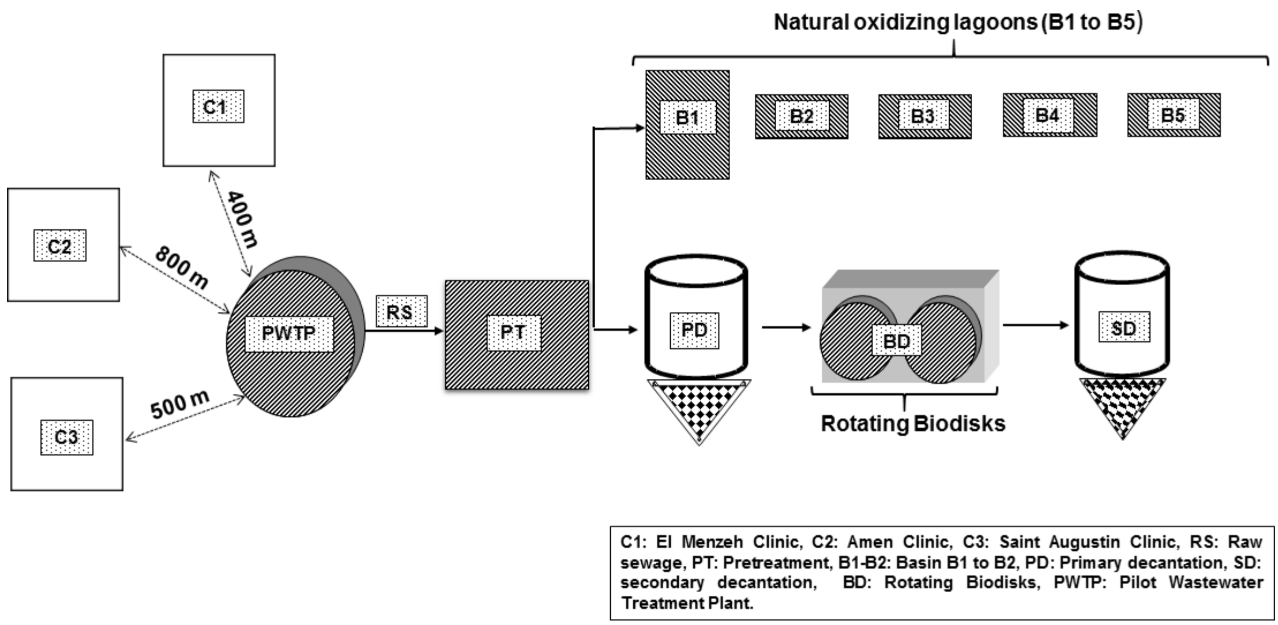

Fig. 1. Location site of pilot wastewater treatment plant and a simple schematic of two biological treatment procedures.

US Environmental Protection Agency, using the adapted method of beef extract and $AlCl_3$ [17–20]. The viral particles were precipitated by polyethylene glycol 6000 (PEG 6000) and rocked at 4°C for 24 h [21]. A filtration using syringe filters of 0.22 μm was used for the viral suspension decontamination.

2.2.2. RNA extraction

Viral RNA was extracted in accordance with the manufacturer's instructions, using an automatic extractor NucliSENS® EasyMag™ platform (bioMérieux, Marcy L'Etoile, France).

2.2.3. Astrovirus detection

HAstVs were detected by reverse transcription polymerase chain reaction (RT-PCR) using the primer sets Mon 244 and Mon 245, to amplify a 413 pb fragment of the gene encoding for the precursor of the ORF2 capsid [22]. The sequences of primer pairs were 5′-GGTGTCACAGGACCAAAACC-3′, 5′-TTAGTGAGCCACCAGCCATC-3′ for Mon 244 and Mon 245, respectively.

RT-PCR was performed using a Qiagen OneStep RT-PCR Kit (France) according to the manufacturer's instructions and to the cycles of amplification given by the authors of each primer set.

The physicochemical characteristics were defined in conformity with the standard method analyses of wastewater in AFNOR [23]. The bacteriological water contents were determined by the enumeration of faecal pollution indicator bacteria (thermotolerant faecal coliforms and faecal Streptococci) using the most probable number method [23].

2.2.4. Sequencing

The sequencing reaction of the purified DNA was achieved with the same primers as those used for amplification (Mon 244/Mon 245) using the ABI Prism® BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, France).

The nucleotide sequences of the amplicons were compared with other sequences of human astroviruses strains available in the GenBank database. Phylogenetic analyses were performed according to the neighbour-joining method with the MEGA software [24] and the PHYLIP package [25]. The phylogenetic tree was visualized with the Tree view program [26]. The nucleotide sequences, found in the present work from wastewater samples, were submitted to GenBank under accession numbers KP636520–KP636526.

2.3. Statistical data analysis

The analysis of variance was done using SPSS software (SPSS for Windows, version 19; SPSS Inc., Chicago, IL, USA). The frequencies of human astroviruses were achieved by the least significant difference test in conformity with the Waller–Duncan a, b test.

3. Results and discussion

Enteric viruses are usually present in environmental waters and stand for the major cause of waterborne infections and outbreaks [3,4]. In Tunisia, no sufficient environmental information on the presence of human astroviruses in wastewater is available. In this survey, the circulation of enteric viruses such as human astroviruses was monitored in the semi-industrial wastewater treatment, pilot plant, in a residential city of Tunis, Tunisia. The wastewater in this area of study is very specific since it is originated from three neighbouring clinics. Therefore, this hospital wastewater could be qualified as specific and particularly contaminated water. Hospital wastewater contains many pollutants such as pathogenic microbes (bacteria, viruses), biodegradable toxic and radioactive contaminants that can cause pollution and health problems [27].

In this study, 56 samples were positive for human astroviruses (55%). Therefore, this work documents for the first time a real investigation of human astroviruses detection in Tunisian hospital wastewater. Previous Tunisian studies on viruses concerned clinic and hospital environments where the human astroviruses were detected in 3.6% and 7% of faecal samples between the years 2003–2005 [13] and 2003–2007 [5], respectively. Similarly, human astroviruses were detected in 61% of Tunisian shellfish samples [14]. Moreover, human astroviruses were not found in wastewater and in shellfish samples in the first Tunisian environmental study between the years 2003 and 2007 [15]. This high detection rate of human astroviruses in the present study is not similar to those registered in other Tunisian studies [5,13–15]. These findings support the relevance of this type of virus as an important factor and major cause of waterborne infectious diseases in children in Tunisia and in the rest of the world. Similarly, human astroviruses have been found in 45% of sewage samples directly discharged into the Uruguay River during 1 year of study [6]. Nevertheless, human astroviruses have been found at various rates in sewage samples from different rural areas worldwide, such as South Africa, Germany, Hungary, Egypt, Italy, Singapore, Brazil and Japan [8,28–35]. The high detection rate of human astroviruses around 55% registered in this work reflects the fact that the human astroviruses circulated at a comparatively high prevalence in the studied local population. This high prevalence may be connected or conditioned by the presence of three important clinics in the neighbourhood. These results demonstrate and confirm that environmental surveillance could be viewed as an effective method to control the epidemiology of human astroviruses and contribute to mastering and understanding diseases caused by human astroviruses in populations.

The molecular detection of human astroviruses in treated effluent samples included in this study revealed that 45 (44%) of the positive wastewater samples were recorded in the five different natural lagoon basins (B1 to B5) from the natural oxidizing lagoon process, and only 11 (11%) were registered at the exit of the rotating biological disk procedure. Therefore, the distribution of the overall frequencies of human astroviruses in the two procedures of treatment was 6% (n = 6/102), 5% (n = 5/102), 12% (n = 12/102), 8% (n = 8/102), 14% (n = 14/102) and 11% (n = 11/102) within the five lagoon basins (B_1–B_5) and at the outlet of the rotating biological disks (D) process; respectively. Within the different lagoon basins (B_1–B_5), the distribution of human astroviruses was used to establish the frequencies of human astroviruses in each lagoon. These frequencies were as follows: 23% (n = 6/26) in B_1, 24%

(n = 5/21) in B_2, 80% (n = 12/15) in B_3, 67% (n = 8/12) in B_4 and 88% (n = 14/16) in B_5. At the exit of the rotating biological disk procedure, 92% (n = 11/12) of the samples were contaminated by human astroviruses (Fig. 2). Thereby, the human astroviruses detection results in all positive samples depicted particularly low frequencies of human astroviruses in the two basins B_1 and B_2 when compared with the three other basins B_3, B_4, B_5 from the natural oxidizing lagoon process and at the termination of the rotating biological disk procedure (D). Human astroviruses were detected only in 23% and 24% of wastewater samples in the two basins B_1 and B_2, respectively. On the other hand, more or less high frequencies of human astroviruses were registered in the three other basins (B_3, B_4, B_5), and at the exit of the rotating biological disks (80% in B_3, 67% in B_4, 88% in B_5 and 92% in the D), when likened with the values found in the two basins B_1 and B_2 of the natural oxidizing pond procedure. In the effluents and influents of the two treatment procedures, the human astroviruses frequencies showed progressively a significant increase of the different frequencies of these viruses recorded in the five basins of natural oxidization ponds and at the exit of the rotating biodisks procedure ($P < 0.05$; Fig. 2; Table 1).

Similarly, human astroviruses are commonly present in environmental waters and represent the major cause of waterborne infections and outbreaks, since the traditional wastewater treatments usually fail to remove enteric viruses in the water purification processes [7]. This fact enhances and promotes the daily release of these types of viruses into natural receiving waters. The present work evaluates two types of biological treatment procedures, namely natural oxidizing lagoons and rotating biological disks situated in a semi-industrial pilot wastewater treatment plant and located in the Tunis City suburbs for their waterborne human astroviruses removal capabilities.

The results obtained in the two types of treatment processes showed mainly a significant increase of the prevalence of human astroviruses from the first to the last lagoon of the five lagoons of natural oxidizing lagoons and at the exit of the rotating biological disks. These data showed the ineffectiveness of the two recommended wastewater treatment methods for the human astroviruses removal. This increase of human astroviruses frequencies could be explained by the phenomenon of disaggregation largely observed with enteric virus particles during their transport through the various lines of treatment. Furthermore, the massive presence of the

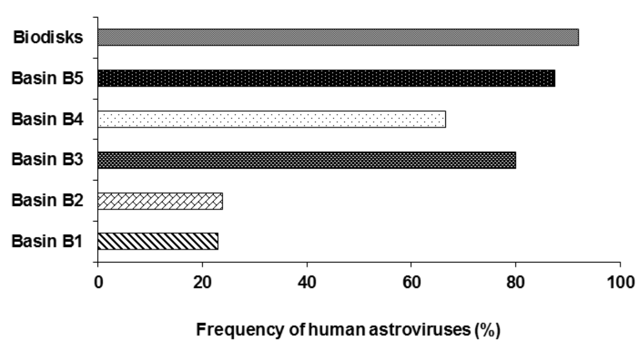

Fig. 2. Annual variation of the frequencies of human astroviruses in the two wastewater treatment procedures.

Table 1
Annual variation of the frequencies of human astroviruses detected at the exit of the two wastewater treatment processes during the year of study

	Frequencies of human astroviruses												
	December	January	February	March	April	May	June	July	August	September	October	November	Total
Basin B_1	2 a; α	2 a; α	0 a; α	0 a; α	1 a; α	0 a; α	0 a; α	0 a; α	0 a; α	0 a; α	0 a; α	1 a; α	6 α
Basin B_2	0 a; α	0 a; α	1 a; α	1 a; α	1 a; α	1 a; α	0 a; α	0 a; α	0 a; α	0 a; α	1 a; α	0 a; α	5 α
Basin B_3	2 a; α	0 a; α	0 a; α	1 a; α	2 a; α	2 a; α	1 a; α	1 a; α	0 a; α	1 a; α	1 a; α	1 a; α	12 α
Basin B_4	2 a; α	0 a; α	0 a; α	1 a; α	2 a; α	1 a; α	0 a; α	1 a; α	0 a; α	1 a; α	0 a; α	0 a; α	8 α
Basin B_5	3 b, c; α	0 a; α	2 a, b, c; α	0 a; α	4 c; α	3 b, c; α	0 a; α	1 a, b; α	0 a; α	1 a, b; α	0 a; α	0 a; α	14 α
Biodisks	2 a; α	1 a; α	2 a	1 a; α	2 a; α	1 a; α	0 a; α	1 a; α	0 a; α	1 a; α	0 a; α	0 a; α	11 α
Total	11 c, d	3 a, b	5 a, b, c	4 a, b	12 d	8 b, c, d	1 a	4 a, b	0 a	4 a, b	2 a, b	2 c, d	56

Note: a, b, c … within a column means followed by the same minuscule letter are significantly different according to Waller–Duncan a, b at $P < 0.05$; α, β … within a line means followed by the same majuscule letter are significantly different according to Waller–Duncan a, b at $P < 0.05$.

human astroviruses particle aggregates in wastewater could in part give an explanation concerning the resistance of these viruses to recommended treatment [36].

The phenomenon of aggregation or clumping of virus particles protect these viruses from some environmental inactivating agents and increase the possibility of complementation between some non-infectious particles [36]. In summation, the adsorption status of viruses to particles or surfaces has been demonstrated to greatly affect virus survival [37]. Several kinds of forces and factors could play an important role in viral adsorption. These various forces and factors may include ionic attractions, covalent reactions with certain chemicals, hydrogen bonding, hydrophobic interactions, double layer interactions, Van der Waals attractions, pH, ionic forces, organic and mineral content, etc. The existing theory of virus adsorption suggests that the most important of these forces are the double-layer interactions, the pH and the Van der Waals attractions, largely and previously described by Gerba [37,38].

Generally, various categories of viruses could persist in natural environments during many hours, days, weeks or even months (liquid, solid and in the airborne state). The period of persistence depends essentially on the type of viruses, their physical state (dispersed, aggregated, cell-associated, membrane-bound, adsorbed to other solids), the medium in which it is present (faeces, respiratory secretions, tissues, other liquids or solids, air), and particularly the prevailing environmental conditions (temperature, pH, organic matter, particulates, salt concentration, protective ions, proteolytic enzymes, antiviral microbial activity, and light). All these parameters seriously influence the virus survival.

The high frequency and persistence of these human astroviruses observed and detected in the treated wastewater could be related concurrently to the initial high number (the dominance) of this kind of virus in the raw wastewater, and to its resistance to the treatment used in this study.

These results indicate the poor sanitary quality of treated wastewater at the release of these two procedures for recycling and reuse in agriculture. These findings document for the first time in Tunisia that has reported the ineffectiveness of biological treatment for human astroviruses removal from wastewater treatment plant. In the same manner, an Egyptian environmental study has identified a high resistance of human astroviruses in the activated sludge process in three different sewage treatment plants [31]. Conversely, two other environmental studies in Brazil and in Italy pointed out the biological treatment effectiveness in removing human astroviruses by two activated sludge procedures [32,39]. The resistance of human astroviruses to the biological wastewater treatment adopted in this study and its persistence in treated wastewater indicated that these biological procedures tested were not efficient to eliminate human astroviruses. This result confirmed the principal role of water, especially wastewater, in the transmission and dissemination of HAstVs by drinking polluted water. This is a major public health concern. Innovative and improved existing treatment processes are required. Our method could be successfully applied in assessing new treatment procedures to decrease the viral load specifically by various techniques of water disinfection. It could also guarantee the prevalence lessening of human astroviruses in the effluent of treatment plants and different receiving water bodies.

Furthermore, the appearance of the studied virus was detected, especially when the wastewater possessed an alkaline pH extended from 7.6 to 8.6 and the ambient temperatures ranged from 10°C to 25.7°C. Thereby, a noticeable peak was observed in the cold seasons. Indeed, important frequencies were recorded in the winter (11% in December) and in the spring seasons (12% in April, 8% in May).

A clear difference was observed during the monthly circulation of the human astroviruses frequencies in the two treatment methods: natural oxidizing lagoons, also called stabilization lagoons or ponds and rotating biological disks ($P < 0.05$; Fig. 3; Table 1).

The studied virus was found, during the three average temperature seasons such as winter, autumn and spring periods with low frequencies in the first two lagoons B_1 and B_2 of natural oxidizing lagoon procedures (2% in December and in November in B_1; 1% in February, March, April, May and October in B_2; Table 1). However, human astroviruses were detected with important frequencies in winter and in the spring seasons, in the three other lagoon basins (B_3, B_4, B_5) and at the exit of the rotating biological disks (D) (3% in December and 4% in April in B_5; Table 1). Results in Table 1 mirrored an important difference in the human astroviruses frequencies over the different seasons only in the lagoon B_5 of the natural oxidizing lagoon process. In contrast, no significant difference in the human astroviruses monthly circulation was observed in the four other lagoons (B1, B2, B3, B4) and at the exit of the rotating biological disks line ($P \leq 0.05$; Table 1).

Amongst the main results of this study, the high prevalence of human astroviruses had been observed during the winter and spring seasons, and the lowest one was observed in summer and autumn, that is, June, August, October and November. Similarly, a peak of human astroviruses detection was observed in the cold months in sewage samples discharged directly into the Uruguay River [6,12]. In discordance with these last results, human astroviruses were identified in wastewater of three treatment plants during the four seasonal campaigns (winter, spring, summer and autumn) in Italy by Anastasi et al. [31]. Similar results on the human astroviruses occurrence in environmental water samples are confirmed by data of Sdiri-Loulizi et al. [5] who reported a

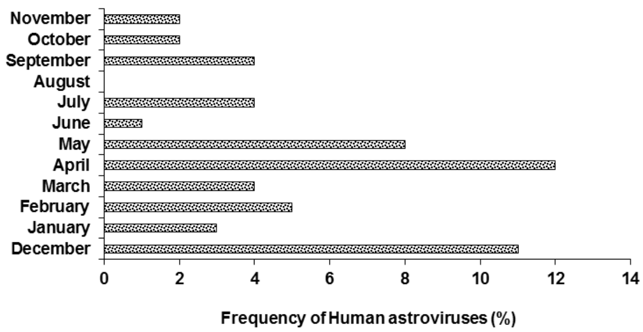

Fig. 3. Monthly variation of the frequencies of human astroviruses in the two wastewater treatment procedures.

continual human astroviruses infection during the year in the Tunisian population. The prevalence of human astroviruses infection is seasonal. Human astroviruses are mostly prevalent in winter in temperate regions [40], but in the tropics, they are predominant in the rainy season [41]. The presence of human astroviruses in the cold seasons was explained by the effect of temperature on the virus survival in the different types of water. In fact, the viral inactivation was faster in warm seasons (summer and fall) when the water temperature is very important.

On the other hand, the results of the physicochemical and bacteriological characteristics in the different lagoon basins from the natural oxidizing lagoon process and at the exit of the rotating biological disk procedure were presented in Table 2. In our survey, we simultaneously analysed different bacteriological parameters in the two treatment procedures (lagoon/pond basins: B1 to B5 and biodisks). Regarding lagoon/pond basin procedures, results demonstrated poor physicochemical quality and good bacteriological quality of processed water. Inversely, for biodisks procedures, results indicated an excellent physicochemical quality and a bad bacteriological quality of processed water. Similar effects were previously identified in other Tunisian studies [16–18]. Indeed, the cited work showed better efficiency to remove main physicochemical pollutants of water and secured efficiency of removing pathogens by natural oxidizing lagoon procedures. Opposite results were found with the rotating biological disk procedure, according to the same survey.

Finally, the molecular typing of human astroviruses strains was conducted on all positive wastewater samples, but only the genotype of seven samples was studied. Fig. 4 presented the phylogenetic tree comparing the Tunisian human astroviruses strains with the other human astroviruses sequences available in GenBank. The dendrogram showed seven viral genotypes and pointed that six Tunisian strains (HAstVTUN1 to HAstVTUN6) belonged to genotype 1 and only one Tunisian strain (HAstVTUN7) belonged to genotype 6 (Fig. 4). The Blast database of the National Centre for Biotechnology Information (NCBI) was used to identify the Tunisian strain by comparison with the closest sequences deposited in GenBank. The phylogenetic tree in Fig. 5 shows the relationship between 64 international sequences of the GenBank Database and the Tunisian strains (Fig. 5). Thereby, the phylogenetic tree illustrated that Tunisian strains were included in two clusters.

The six Tunisian strains revealed in this study (HAstVTUN1 to HAstVTUN6) and belonging to genotype 1 showed a similar nucleotide sequence of 100% with the reference strains JN799267, JN799266, KF156804, JF929185, JX083274, JN799271, KC285161 and JF491425. The Tunisian strain (HAstVTUN7) represented a similar nucleotide sequence of 98%–100% with the reference strains isolated from faecal samples, such as FJ755390 in China [5], GQ901902 in Russia, KF420154 in Bangladesh [11], KC896119 and KC896117 in Pakistan [42], AF292077 in South Africa [43], Z46658 in Oxford region [44], L38507 [22], DQ917382 in Brazil [45] and from surface waters such as HM209123 in Singapore [32] (Fig. 4).

In fact, the two Tunisian strains, HAstVTUN1 and HAstVTUN2, were clustered together in the same cluster and were close to the strain KC285184 isolated in Russia. These last two Tunisian strains HAstVTUN3 and HAstVTUN4 appeared very similar and they are grouped in the same cluster with the two other Tunisian strains HAstVTUN5 and HAstVTUN6 included within the majority of international sequences determined in Brazil and in Russia. In Brazil, these sequences were found in faecal samples (JQ260760, JQ260759) and in sewage samples (JN799266, JN799267 and JN799271) [34,46]. In addition, in Russia, the sequences JX083274, KC285161, JN203051, JF491425 and JF929185 were noticed in faecal samples [47] (Fig. 5).

In the present environmental study, two genotypes (HAstV-1 and HAstV-6) of HAstV were identified in wastewater samples showing the predominance of genotype 1 (n = 6). In a previous Tunisian clinical study conducted by Sdiri-Loulizi et al. [5] reported the detection of two genotypes (HAstV-1 and HAstV-3). In addition, the HAstV-1 genotype was recognized as the most frequent one in Tunisia. Consequently, these data obtained in the present work indicated the detection of HAstV-6 genotype for the first time

Table 2
The results of physicochemical and bacteriological parameters obtained at the exit of natural oxidizing lagoons and rotating biodisks

Parameters/values	Natural oxidizing lagoons (B1–B5)		*P* (%)	Rotating biodisks (D)		*P* (%)
	Minimum	Maximum		Minimum	Maximum	
pH	7.6	8.6		7.6	8.6	
Temperature	10	25.7		10	25.7	
EC (S/cm)	1,720	1,350	–	1.4	1.7	99
SS (mg/L)	58	60	64	26	40	–
COD (mgO_2/L)	155	240	60	105	140	62
BOD_5 (mg/L)	90	170	68	30	50	85
N–NH4 (mg/L)	18	26	52	8.5	19	70
P–PO_4 (mg/L)	17	32	–	11	17	60
FS (NPP/100 mL)	1.6×10^4	1.6×10^6	>99.9	3.2×10^5	5.6×10^6	>90
FC (NPP/100 mL)	3.9×10^3	2.5×10^5		6×10^4	6×10^5	

Min: minimum values; Max: maximum values; EC: electrical conductivity; BOD_5: biological oxygen demand; COD: chemical oxygen demand; SS: suspended solids; NH_4–N: ammonium nitrogen; P–PO_4: *ortho*-phosphate; FS: faecal Streptococci; FC: faecal coliforms; P: performance of treatment; –: not detected.

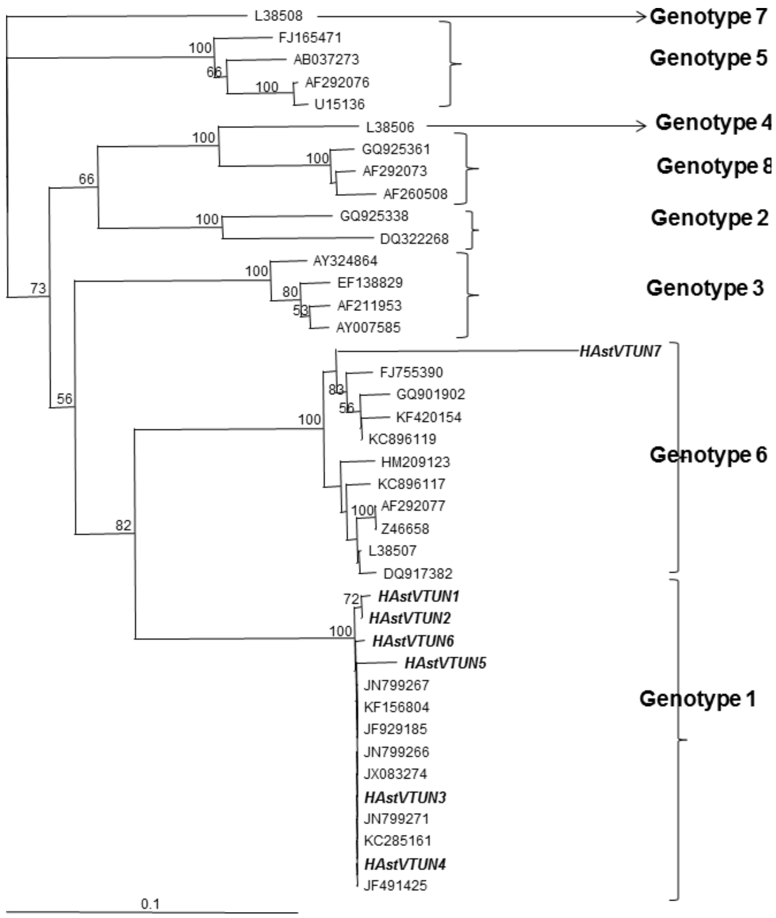

Fig. 4. Phylogenetic trees of human astroviruses based on comparison of sequences in the ORF2 from the seven viral strains isolated in Tunisia and some published nucleotide sequences in GenBank of different genotype of astroviruses. The tree was constructed using neighbour-joining method. Numbers on the branches show bootstrap statistical analysis values (100 replicates). Tunisian sequences reported in the present study are in bold italic.

in Tunisia and confirmed the predominance of HAstV-1 in wastewater sampled in the area of study. Additionally, the two genotypes (HAstV-1 and HAstV-6) of human astroviruses showed a high resistance to two biological procedures of treatment represented by natural lagoon and biodisks line. Therefore, this work exemplifies the first work to investigate the genetic constitution and the epidemiology of human astroviruses via sewage surveillance in Tunisia. The main results obtained were in concordance with those reported in a previous environmental study in Egypt, presenting the emergence of HAstV-6 and HAstV-7 genotypes in the region and the resistance of these genotypes of human astroviruses to secondary and tertiary treatments [30]. In addition, it has been observed that human astroviruses type 1 was the predominant genotype in wastewater samples in Hungary, in Singapore and in Uruguay [8,12,32]. Moreover, other human astroviruses types were brought out in wastewater samples such as HAstV-1, HAstV-2, HAstV-4 and HAstV-5 revealing the predominance of HAstV-1 and HAstV-5 in China [12]. In a recent survey, four cases of classic human astroviruses (HAstV-1, HAstV-2, HAstV-5 and HAstV-4/-8) were detected in sewage samples in Japan [35]. This study constitutes the first report in Tunisia that describes the phylogenetic diversity and genotype distribution of human astroviruses strains circulating in the Tunis region, showing a high frequency and the appearance of new genotypes (HAstV-6) in the country.

4. Conclusion

This work showed the first detection of human astroviruses in hospital wastewater samples directly sampled in a plant for semi-industrial wastewater treatment. This plant located in a residential area and irrigated with wastewater originating amongst others from three neighbouring clinics. The molecular characterization of human astroviruses showed the predominance of HAstV-1 genotype and the identification of HAstV-6 genotype in the region of Tunis City, Tunisia for the first time. Therefore, this work presents the first documentation in Tunisia showing the high resistance of HAstV-1 and HAstV-6 genotypes to two different biological treatments. This work proved the ineffectiveness of the two biological and secondary treatment procedures recommended in this field such as natural oxidizing lagoons and rotating biodisks, for the human astroviruses removal. Thus, tertiary treatment, for example, by using the UV-C_{254}

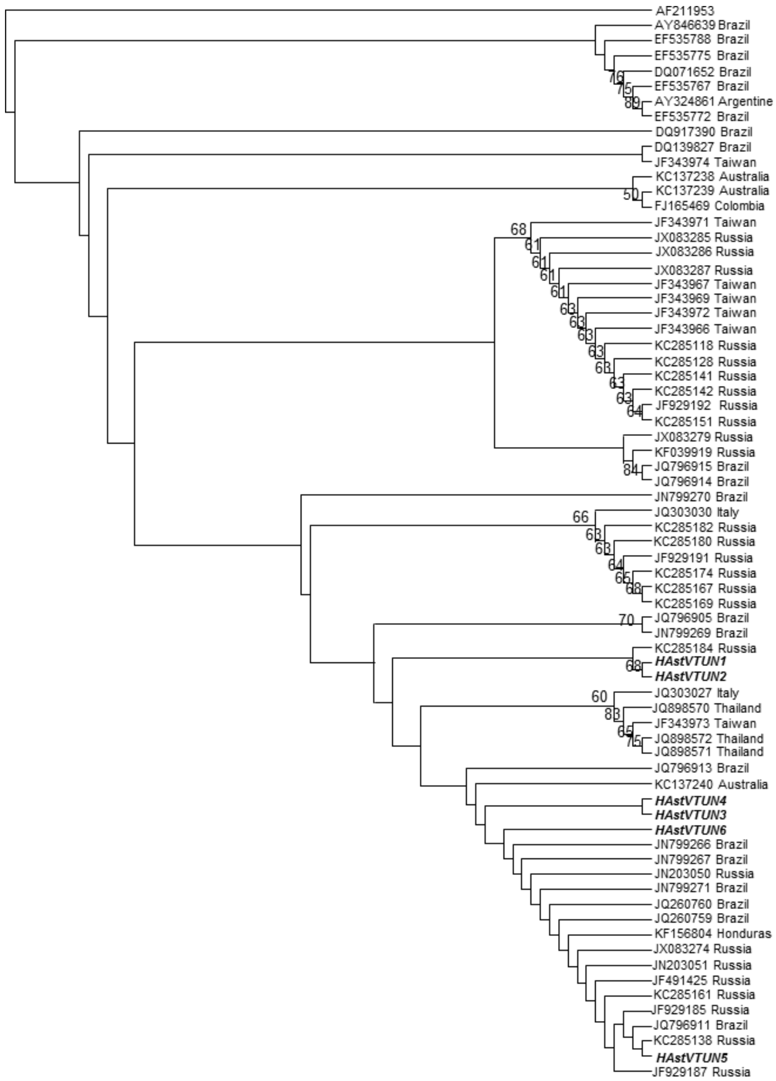

Fig. 5. Phylogenetic trees of human astroviruses based on comparison of sequences in the open reading frame (ORF2) from the six viral strains having genotype 1 detected in Tunisia and 64 published nucleotide sequences in GenBank from different countries. The tree was constructed using neighbour-joining method and the type 3 (AF211953) sequence as the out-group. Numbers on the branches show bootstrap statistical analysis values (100 replicates). Tunisian sequences reported in the present study are in bold italic.

irradiation could be and might be required for the removal of enteric viruses such as human astroviruses [48].

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Conflict of interest

The authors declare that they have no conflicts of interests.

References

[1] I. Di Bartolo, E. Pavoni, S. Tofani, M. Consoli, E. Galuppini, M.N. Losio, F.M. Ruggeri, G. Varisco, Waterborne norovirus outbreaks during a summer excursion in Northern Italy, New. Microbiol., 38 (2015) 109–112.

[2] K. Wazny, A. Zipursky, R. Black, V. Curtis, C. Duggan, R. Guerrant, M. Levine, W.A. Petri, M. Jr. Santosham, R. Scharf, P.M. Sherman, E. Simpson, M. Young, Z.A. Bhutta, Setting research priorities to reduce mortality and morbidity of childhood diarrheal disease in the next 15 years, PLoS. Med., 10 (2013) e1001446.

[3] A.J. Butler, M.K. Thomas, K.D. Pintar, Expert elicitation as a means to attribute 28 enteric pathogens to foodborne, waterborne, animal contact, and person-to-person-transmission routes in Canada, Foodborne. Pathog. Dis., 12 (2015) 335–344.

[4] L. Lu, R. Jia, H. Zhong, M. Xu, L. Su, L. Cao, Z. Dong, N. Dong, J. Xu, Molecular characterization and multiple infections of rotavirus, norovirus, sapovirus, astrovirus and adenovirus in outpatients with sporadic gastroenteritis in Shanghai, China, 2010–2011, Arch. Virol., (2015) 1229–1238.

[5] K. Sdiri-Loulizi, H. Gharbi-Khelifi, A. de Rougemont, M. Hassine, S. Chouchane, N. Sakly, P. Pothier, M.N. Guédiche, M. Aouni, K. Ambert-Balay, Molecular epidemiology of human astrovirus and adenovirus serotypes 40/41 strains related to acute diarrhoea in Tunisian children, J. Med. Virol., 81 (2009) 1895–1902.
[6] M. Victoria, L.F. Tort, M. García, A. Lizasoain, L. Maya, J.P. Leite, M.P. Miagostovich, J. Cristina, R. Colina, Assessment of gastroenteric viruses from wastewater directly discharged into Uruguayan River, Uruguay, Food Environ. Virol., 6 (2014) 116–124.
[7] F. Sezen, E. Aval, T. Ağkurt, Ş. Yilmaz, F. Temel, R. Güleşen, G. Korukluoğlu, M.B. Sucakli, M.A. Torunoğlu, B.P. Zhu, A large multi-pathogen gastroenteritis outbreak caused by drinking contaminated water from antique neighbourhood fountains, Erzurum City, Turkey, December 2012, Epidemiol. Infect., 143 (2015) 704–710.
[8] E. Meleg, F. Jakab, B. Kocsis, K. Bácnyai, B. Melegh, G. Szcs, Human astroviruses in raw sewage samples in Hungary, J. Appl. Microbiol., 101 (2006) 1123–1129.
[9] E. Mendez, C.F. Arias, Astroviruses, D.M. Knipe, P.M. Howely, Fields Virology, 5th ed., Lippincott Williams and & Wilkins, Philadelphia, 2007, pp. 981–1000.
[10] S. De Grazia, M.A. Platia, V. Rotolo, C. Colomba, V. Martella, G.M. Giammanco, Surveillance of human astrovirus circulation in Italy 2002–2005: emergence of lineage 2c strains, Clin. Microbiol. Infect., 17 (2011) 97–101.
[11] M.H. Afrad, P.C. Karmakar, S.K. Das, J. Matthijnssens, F. Ahmed, S. Nahar, A.S. Farugue, M. Rahman, T. Azir, Epidemiology and genetic diversity of human astrovirus infection among hospitalized patients with acute diarrhoea in Bangladesh from 2010 to 2012, J. Clin. Virol., 58 (2013) 612–618.
[12] A. Lizasoain, L.F. Tort, M. García, M.M. Gómez, J. Cristina, J.P. Leite, M.P. Miagostovich, M. Victoria, R. Colina, Environmental Assessment of Classical Human Astrovirus in Uruguay, Food. Environ. Virol., 7 (2015) 142–148.
[13] I. Fodha, A. Chouikha, J. Dewar, A. Trabelsi, N. Boujaafar, A.D. Steele, Prevalence of adenovirus antigens in children presenting with acute diarrhoea, Med. Trop., 67 (2006) 256–258.
[14] D.E. Elamri, M. Aouni, S. Parnaudeau, F.S. Le Guyader, Detection of human enteric viruses in shellfish collected in Tunisia, Lett. Appl. Microbiol., 43 (2006) 399–404.
[15] K. Sdiri-Loulizi, M. Hassine, Z. Aouni, H. Gharbi-Khelifi, S. Chouchane, N. Sakly, M.N. Guédiche, P. Pothier, M. Aouni, K. Ambert-Balay, Detection and molecular characterization of enteric viruses in environmental samples in Monastir, Tunisia between January 2003 and April 2007, J. Appl. Microbiol., (2010) 1093–1104.
[16] A. Hassen, N. Jedidi, H. Kallali, M. Ferchichi, A. Ghrabi, F. Chebbi, N. Saidi, H. Shayeb, M. Ennabli, Élimination des indicateurs et des métaux lourds au cours de traitement des eaux usées domestiques dans une station pilote semi-industrielle, Sci. Techn. de l'eau., 27 (1994) 34–41.
[17] C. Ibrahim, N. Chérif, S. Hammami, P. Pothier, A. Hassen, Quantification and molecular characterization of Norovirus after two wastewater treatment procedures, Water Air Soil Pollut., 226 (2015) 187–200.
[18] C. Ibrahim, N. Chérif, S. Hammami, P. Pothier, A. Hassen, Quantification and genotyping of Rotavirus A within two wastewater treatment processes, J. Clean Soil. Air. Water, 44 (2016) 393–401.
[19] Environmental Protection Agency, Standards for the Disposal of Sewage Sludge, In Federal Register Part, 503, 1992, pp. 9387–9404.
[20] Environmental Protection Agency, Environmental Regulations and Technology, Control of Pathogens and Vector Attraction in Sewage Sludge, Including Domestic Septage, Under 40 CFR, Part, 503, 2003, pp. 625–692.
[21] G.D. Lewis, T.G. Melcaft, Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and Human Rotavirus, from oyster, water and sediment samples, Appl. Environ. Microbiol., 54 (1988)1983–1988.
[22] J.S. Noel, T.W. Lee, J.B. Kurtz, R.I. Glass, S.S. Monroe, Typing of Human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing, J. Clin. Microbiol., 33 (1995) 797–801.
[23] J. Rodier, L'analyse de l'eau, Eaux naturelles, eaux résiduaires, eaux de mer, 6th Édn Dunod Paris ISBN: 2100496360, 1978, pp. 1383.
[24] S. Kumar, K. Tamura, M. Nei, MEGA: Molecular Evolutionary Genetics analyses software for microcomputers, Comput. Appl. Biosci., 10 (1994) 189–191.
[25] J. Felsenstein, PHYLIP (Phylogeny Inference Package) Version 3.5.c. Department of Genetics, University of Washington, 1993. Available at:http://www.Caos.kun.nl/cammsa/PHYLIP.
[26] R.D.M. Page, TREEVIEW: An application to display phylogenetic trees on personal computers, Comput. Appl. Biosci., 12 (1996) 357–358.
[27] V.P. Prabhasankar, D.I. Joshua, K. Balakrishna, I.F. Siddiqui, Removal rates of antibiotics in four sewage treatment plants in South India, Environ. Sci. Pollut. Res. Int., 23 (2016) 8679–8685.
[28] S. Nadan, J.E. Walter, W.O. Grabow, D.K. Mitchell, M.B. Taylor, Molecular characterization of astroviruses by reverse transcriptase PCR and sequence analysis: comparison of clinical and environmental isolates from South Africa, Appl. Environ. Microbiol., 69 (2003) 747–753.
[29] D. Pusch, D.Y. Oh, S. Wolf, R. Dumke, U. Schröter-Bobsin, M. Höhne, I. Röske, E. Schreier, Detection of enteric viruses and bacterial indicators in German environmental waters, Arch. Virol., 150 (2005) 929–947.
[30] W. Morsy El-Senousy, S. Guix, I. Abid, Pintó, A. Bosch, Removal of astrovirus from water and sewage treatment plants, evaluated by a competitive reverse transcription-PCR, Appl. Environ. Microbiol., 73 (2007) 164–167.
[31] P. Anastasi, E. Bonanni, G. Cecchini, M. Divizia, D. Donia, F. Di Gianfilippo, R. Gabrieli, A.R. Petrinca, A. Zanobini, Virus removal in conventional wastewater treatment process, Iq. Sanita. Pubbl. 64 (2008) 313–330.
[32] T.G. Aw, K.Y. Gin, Environmental surveillance and molecular characterization of human enteric viruses in tropical urban wastewater, J. Appl. Microbiol., 109 (2010) 716–730.
[33] X.Q. He, L. Cheng, D.Y. Zhang, X.M. Xie, D.H. Wang, Z. Wang, One-year monthly survey of rotavirus, astrovirus and norovirus in three sewage treatment plants (STPs) in Beijing, China and associated health risk assessment, Water. Sci. Technol., 64 (2011) 1202–1210.
[34] T.M. Fumian, C.B. Vieira, J.P. Leite, M.P. Miagostovich, Assessment of the burden of virus agents in an urban sewage treatment plant in Rio de Janeiro, Brazil, J. Water. Health., 11 (2013) 110–119.
[35] A. Hata, H. Katayama, M. Kitajima, H. Furumai, Wastewater analysis indicates that genetically diverse Astroviruses, including strains belonging to novel clades MLB and VA, are circulating within Japanese populations, Appl. Environ. Microbiol., 81 (2015) 4932–4939.
[36] D. Roy, R.S. Engelbrecht, P.K.Y. Wong, E.S. Chian, Inactivation of enteroviruses by ozone, Appl. Environ. Microbiol., 41 (1981) 718–723.
[37] C.P. Gerba, Applied and theoretical aspects of virus adsorption to surfaces, Advances. Appl. Microbiol., 30 (1984) 133–168.
[38] C.P. Gerba, Risk Assessment, R.N. Maier, I.L. Pepper, C.P. Gerba, Environmental Microbiology, 2 ed., Burlington, Academic Press/Elsevier, 2008, pp. 598.
[39] F.R. Guimarães, F.F. Ferreira, C.B. Vieira, Fumian, T. Shubo, J.P. Leite, M.P. Miagostovich, Molecular detection of human astrovirus in an urban sewage treatment plant in Rio de Janeiro, Brazil, Mem. Inst. Oswaldo. Cruz., 103 (2008) 819–823.
[40] S. Guix, S. Cabellero, C. Villena, R. Bartolomé, C. Latorre, N. Rabella, M. Simó, A. Bosch, R.M. Pintó, Molecular epidemiology of Astrovirus infection in Barcelona, Spain, J. Clin. Microbiol., 40 (2002) 133–139.
[41] T.A. Nguyen, L. Hoang, D. Pham le, K.T. Hoang, M. Mizuguchi, S. Okitsu, H. Ushijima, Identification of human astrovirus infections among children with acute gastroenteritis in the Southern Part of Vietnam during 2005–2006, J. Med. Virol., 80 (2008) 298–305.
[42] M.M. Alam, A. Khurshid, M.S. Rana, S. Shaukat, S. Sharif, M. Angez, M. Naeem, S.S. Zaidi, Serotype diversity of astroviruses in Rawalpindi, Pakistan during 2009–2010, PLoS. One., 8 (2013) E61667.

[43] J.E. Walter, J. Briggs, M.L. Guerrero, D.O. Matson, L.K. Pickering, G. Ruiz-Palacios, T. Berke, D.K. Mitchell, Molecular characterization of a novel recombinant strain of human astrovirus associated with gastroenteritis in children, Arch. Virol., 146 (2001) 2357–2367.

[44] T.W. Lee, J.B. Kurtz, Prevalence of human astrovirus serotypes in the Oxford region 1976–92, with evidence for two new serotypes, Epidemiol. Infect., 112 (1994) 187–193.

[45] Y.B. Gabbay, A.C. Linhares, E.L. Cavalcante-Pepino, L.S. Nakamura, D.S. Oliveira, L.D. da Silva, J.D. Mascarenhas, C.S. Oliveira, T.A. Monteiro, J.P. Leite Prevalence of human astrovirus genotype associated with acute gastroenteritis among children in Belem, Brazil, J. Med. Virol., 79 (2007) 530–538.

[46] M.S.R. Ferreira, M.T.P. Xavier, A.C.C. Tinga, T.L. Rose, T.M. Fumian, A.M. Fialho, R.M. Assis, F.A. Carvalho-Costa, S.A. Oliveira, J.P.G. Leite, M.P. Miagostovich, Assessment of gastroenteric viruses frequency in a children's day-care centre in Rio de Janeiro, Brazil: a fifteen-year study (1994–2008), PloS. One., 7 (2012) e33754.

[47] A.T. Podkolzin, T.A. Konovalova, M.L. Lakovenko, S.I. Braslavskaia, G.A. Shipulin, Astroviral infection in Russian Federation, Vopr. Viru. Sol., 58 (2013) 32–37.

[48] V.J. Harwood, A.D. Levine, T.M. Scott, V. Chivukula, J. Lukasik, S.R. Farrah, J.B. Rose, Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection, Appl. Environ. Microbiol., 71 (2005) 3163–3170.