Original Article

Molecular Epidemiology of Anellovirus Infection in Children's Urine: A Cross-sectional Study

Abstract

Background: Anelloviridae is a viral family which is considered as a constant component of human virome. Given the ubiquitous nature of the virus infection and the long-standing relationship between the virus and the host, in the present study, we aimed at investigating the presence of Anelloviruses in the urine samples of children in a cross-sectional study. **Materials and Methods:** The urine samples of 50 children who were referred to Hazrat Ali Asghar Children's Hospital, affiliated to Iran University of Medical Sciences, Tehran, Iran, were obtained. Three TaqMan real-time polymerase chain reactions (PCRs) were carried out for Anellovirus detection. A phylogenetic tree was drawn for positive products after PCR amplification, purification, and nucleotide sequencing. SPSS, version 20, was used for statistical analyses. **Results:** Children's mean age ± standard deviation was 4.30 ± 1.47 years and 56% (28/50) were female. Real-time PCR revealed that Anellovirus was positive in 12% (6/50). Furthermore, PCR-sequencing results showed that torque teno virus was detected in 83.3% (5/6) and SEN virus in 16.6% (1/6) of the Anellovirus positive samples. In addition, 86% (5/6) of the children with positive samples were female. No significant difference was detected between any of the demographic characteristics and Anellovirus positivity ($P > 0.05$). **Conclusion:** According to our preliminary study, the presence of Anelloviruses in the urine samples of asymptomatic children in Iran is striking, although limited sample size and age range limitations might have affected the comprehensive results of our study.

Keywords: *Anellovirus, viral infection, virome*

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Introduction

The development of molecular methods during recent years has helped researchers to show that the urine obtained from healthy individuals can be colonized by nonpathogenic viruses and bacteriophages.[1] The role of nonpathogenic viruses in human physiology has not fully been elucidated. Many studies have shown evidence of their impact on disease susceptibility and immune system evolution.[2] Due to the extensive existence of Anelloviridae infection in nature, its resistance to antiviral drugs, and its effect on the immune systems, monitoring these viruses in the virome of different communities seems necessary.[3] Anelloviridae is a family of nonenveloped viruses with a capsid containing 12 pentameric capsomeres into an icosahedral symmetry. They have a circular single-stranded DNA genome with a negative polarity and their genome has coding and noncoding regions. The coding region has four open reading frames (ORFs). ORF1 contains hypervariable regions that are responsible for wide range of genetic variations.[4] Based on ORF1, the International Committee on Taxonomy of Viruses (ICTVs) defines three genera for human Anelloviruses, including Alpha Torque Teno, Beta Torque Teno, and Gamma Torque Teno viruses. The prototype of this family was isolated by Nishzawa *et al*. in 1997 and was called "Torque Teno virus" (TTV). Then, other viruses of this family such as Torque Teno Midi virus (TTMDV) and Torque Teno Mini virus (TTMV) were isolated in 2000 and 2007, respectively. In addition, the SEN virus (SENV) is a single-stranded DNA virus that is identified as a member of the TTV family.[5] In the lack of suitable cell culture system and serological tests, the common detection method for TTV is based on molecular techniques.[6] No specific tissue tropism is known for these viruses.

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Replication of these viruses occurs in various tissues, such as the liver, lymph nodes, spleen, lung, muscles, lymphocytes, and polymorphonuclear cells.[7] However, the main replication site of the virus has been reported to be in T-cell lymphocytes[4] and the replication of the virus may be performed by rolling circle mechanisms.[8]

The prevalence of TTVs is different in different countries worldwide, for instance, about 5% in Brazil and 90% in Russia and Japan. The prevalence of TTVs in the Iranian population has been estimated as between 26% and 96%.[9-12]

TTV transmissions through blood transfusion, saliva, and respiratory secretions were documented. Moreover, sexual and vertical transmissions from mother to the fetus have been other transmission routs recognized.[11] TTVs do not cause a serious disease. They may affect cytokine hemostasis and host immune response and can cause autoimmune diseases.[13] Due to the TTV infection cycle, Spandole *et al*. used the term "Commensal" for Anelloviridae.[11] Furthermore, Anelloviruses could be considered as a constant component of human virome, and we also know that TTV prevalence is different in various populations.[13] Therefore, the present study was conducted to determine the presence of Anellovirus in the urine samples of children so as to examine the children's urine virome and to monitor their immune system.

Materials and Methods

Urine samples were obtained from 50 children who were referred for routine periodic check and were negative for bacterial and fungal infections using standard protocols. Eligible samples were randomly selected from hospital outpatient visits. The samples were collected from October 2017 to February 2018 using pediatrician and children's health records of Hazrat Ali Asghar Children's Hospital, affiliated to Iran University of Medical Sciences, Tehran, Iran. Ethics Committee of Iran University of Medical Sciences, Tehran, Iran, approved the research protocol (No. IR.IUMS.FMD.REC 1396.9223479201). Furthermore, written informed consent was obtained from each participant or his/her parent/guardian. They were informed about our nonaggressive procedures to assent the study protocol. The children did not have any underlying conditions, such as diabetes mellitus, heart disease, infectious diseases, and immune system deficiency, according to the existing data and medical laboratory repository records. The children had no symptoms of lower urinary tract infection, and their urine culture tests were negative for bacterial infection. All specimens, based on the microbiological guideline, had colony-forming unit $<10^3$ and leukocyte per high powered field <10. Hematuria and proteinuria were not recorded for the collected samples. Samples with the results of pyuria and hematuria, as symptoms of fungal infection of the urinary tract, were excluded from the study. Urine samples obtained with catheterization were not used to prevent candida infection.[14-16]

Sterile urine culture containers were used for sample collection and were then stored at − 20°C. DNA extraction was carried out using the MagPurix 12S machine (MagPurix® 12S, Resnova, Rome, Italy) based on magnetic embedding technique making use of Magpurix viral nucleic acid extraction kit (ZPO2003). Furthermore, evaluation of the quality of the isolated DNA was performed via NanoDrop ND-1000® (Thermo Fisher Scientific Inc., Waltham, MA, USA) spectrophotometry.

Real-time polymerase chain reaction

A TaqMan real-time polymerase chain reaction (PCR) was performed for the detection of untranslated region (UTR) of TTV, TTMDV, and TTMV, separately. Primers and probes were obtained from previous studies. Table 1 shows the primers and probes sequences used in the present study. By these primers, Alpha TTV, Beta TTV, and Gama TTV could be detected distinctively. The primers used in the current study for testing were very accurate and were based on the primers described by Takashi *et al*. These primers are designed for the UTR of the viral genome.

For real-time PCR, Rotor-Gene 6000 (Rotor gene-Q, Hilden, Germany) instrument was used. The total volume of 15 μL reaction mixture consisted of 7.5 μL of 2 × TaKaRa TaqMan RT-PCR mix (TaKaRa, Shiga, Japan), 0.5 μM concentration of each forward and reverse primers and probe, 0.2–0.5 μM concentration of template, and the rest of the total volume was obtained by adding distilled water. The heating program was 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, and 30 s at 60°C. The acquisition was performed in annealing/extension step.

Conventional polymerase chain reaction

Each specimen was confirmed and rechecked using a conventional PCR, and the PCR products were sequenced

Table 1: Primer and probe sequences used in our study

Virus	Type	5'-3'
TTV	Forward	CAC TTC CGA ATG GCT GAG TT
	Reverse	GCC TTG CCC ATA GCC CGG
	Probe^	TCCCGAGCCCGAATTGCCCCT
TTMDV	Forward	GTT TTC CAC GCC CGT CCG C
	Reverse	AGA GCC TTG CCC ATA GCC
	Probe^	TCA AGG GGC AAT TCG GGC T
TTMV	Forward	AGT TTA TGC CGC CAG ACG
	Reverse	CCC TAG ACT TCG GTG GTT TC
	Probe^	ACT CAC CTT CGG CAC CCG C
cPCR*	Forward	TWCYCMAACGACCAGCTAGACCT
	Reverse	GTTTGTGGTGAGCAGAACGGA

^All probes were labeled by FAM and TAMRA fluorescent/quencher (s), *cPCR primers detecting variable region of Anelloviruses for genotyping. TTV: Torque Teno virus, TTMDV: Torque Teno Midi virus, TTMV: Torque Teno Mini virus, cPCR: Conventional polymerase chain reactions,

for further analysis. The total volume of 50 µl of reaction mixture contained 0.2-0.5 µM template or control, 25 µl of 2 × Amplicon III mix (Odense M, Denmark), 0.5 µM of each primer, and sterilized distilled water to achieve the total volume. Appropriate positive and negative controls (not template specimen) were used for reaction standardization. PCR was carried out using Bio-Rad T100™ Thermal cycler and the following program: 1 cycle of 5 min at 95°C, 40 cycles of 1 min at 95°C, 1 min at 56°C, 1 min at 72°C; and 1 cycle of 10 min at 72°C. Furthermore, visualizing the PCR products was performed via UV radiation emitted by E-Box Vilber version 15.03 (Vilber Lourmat, ZAC de Lamirault, France) gel documentation system after gel electrophoresis into a 1.5% agarose gel concentration.

Nucleotide sequencing

ExoSAP-IT reagent (USB Corporation, Cleveland, Ohio) was used for the purification of PCR products according to the manufacturer's protocol. A bidirectional sequencing was performed via ABI 370 XL sequencer (Thermo Fisher Scientific Life Technologies Corporation). Raw data were trimmed and analyzed using CLC Workbench 5.0, and reference sequences as shown in Figure 1. After the primary analysis, DNA sequences were compared with Anelloviridae sequences found in NCBI using online BLAST software (http://blastonline.nih.gov/blast). Then, MEGA version 6 was used to draw a phylogenetic tree using neighbor-joining method.

Statistical analysis

SPSS software, version 20 (SPSS Inc., Chicago, IL, USA), was used for evaluating the association between different demographic and pathological variables using relevant statistical tests. Chi-square test was run to compare qualitative variables, and Fisher exact test or Student's *t*-test was used for numerical variables calculation. $P < 0.05$ was considered as statistically significant.

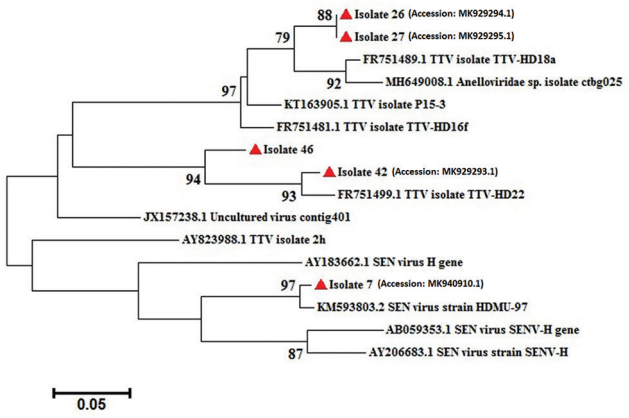

Figure 1: Phylogenetic tree of our Anellovirus-positive isolates by neighbor-joining method. Bootstrap after 1000 replicates calculated and values under 70 were not shown

Results

Samples

A total of 50 urine samples were obtained from children with negative bacterial urine culture results. The children (mean age ± standard deviation [SD] 4.30 ± 1.47 years; mean age ± SD: 4.46 ± 1.40 years) had no underlying illnesses or immune deficiency, and 56% (28/50) of them were female.

Real-time polymerase chain reaction

From among the 50 urine samples tested via three primer pairs and probes, six (12%) specimens were positive for Anellovirus infection. TTV was detected in 83% (5/6) of the positive samples and SENV was detected in 17% (1/6).

The mean age ± SD of the six children with positive samples was 5.50 ± 1.02 years ($P = 0.094$), and three (50%) were in the age range of 6–8 years and five children were female (86.6%). The frequency of infection in the female group was significantly higher although other variables were not significant. More details are given in Table 2.

A conventional PCR was performed by general primers for Anelloviridae family and five were sequenced based on the quality. Raw sequences were analyzed using bioinformatics software. The phylogenetic tree showed that four sequenced isolates had high similarity with Germany and USA submitted sequences and our SENV isolate had high similarity with the submitted sequence from France [Figure 1].

Discussion

During recent years, the study of the human microbiome has received much attention. Virome, as a component of the human microbiome, consists of pathogenic and nonpathogenic viruses. Up to now, the role of nonpathogenic viruses infecting various organs in healthy humans is not clearly understood. In the meantime, Anelloviridae, as a high prevalent virus in different human populations, is a good candidate to investigate human virome. Recent studies have revealed the role of this virus in the stability of the human immune system.[1,2,12]

Anelloviridae was first identified as the cause of posttransfusion hepatitis in 1997.[17] The nonpathogenicity

Table 2: Demographic characteristics of the participants

Variables	Male, *n* (%)	Female, *n* (%)	Total, *n* (%)	*P*
Cases *n* (%)	22 (44)	28 (56)	50 (100)	-
Mean age±SD (years)	3.68±1.55	4.46±1.40	4.30±1.47	0.081
Anellovirus positive				
Total cases	1 (4.5)	5 (17.8)	6 (12)	0.16
Age groups (years)				
4-6	1 (33.3)	2 (66.7)	3 (50)	0.64
6-8	0	3 (100)	3 (50)	0.09

SD: Standard deviation

of the virus has been its crucial feature that has been studied more. The virus is currently a part of the human virome. However, due to the ubiquity of viruses and the lack of a suitable virus culture medium, the pathogenicity of Anelloviridae has not completely been ruled out.[2,3,11] In previous studies, the effect of the virus on the immune system has been shown in healthy individuals.[18] The present study was conducted to determine the prevalence of Anellovirus infection in asymptomatic children's urine as a part of the human virome, but our data failed to find any immune-deficient patient having Anellovirus-positive isolates although inclusion criteria possibly played a role in this. Different studies have reported the presence of the virus in healthy people and the various prevalence rates of Anelloviridae in different communities may be due to the development of molecular methods in recent years.[12,19,20] Similarly, in the current study, the prevalence of Anelloviruses in urine samples of asymptomatic children was found to be 12% (6/50). In a study of kidney transplant recipients in Iran, TTV was detected in 34.2% of the participants. In this study, which was performed on blood samples using semi-nested PCR, a correlation was observed between complications after kidney transplantation similar to urinary tract infection and TTV infection.[21] Furthermore, TTV has been reported as one of the infectious agents of the progressive multiple sclerosis.[22] TTV was isolated in blood samples of hemodialysis patients, fulminant hepatitis, cirrhosis patients, and injection drug addicts.[23-27] In all these studies, the presence of the TTV in healthy populations was reported between 36.7% and 63%. Due to the presence of the virus in all healthy groups, no distinctive link can be found between the pathogenesis of any disease and Anellovirus presence.[21,24,25]

SENV belongs to unclassified Anelloviridae according to ICTV. SENVs is a group of single-stranded DNA viruses that are distantly linked to the TTV virus.[5,28] In a study by Hosseini and Bouzari, the prevalence of SENV has been reported as 90% in Iranian healthy people. In addition, no association has been found between age and sex and the prevalence of SENV in the previous studies.[29] Based on the findings of the present study, SENV was detected in 16.6% of samples (1/6) although Hosseini and Bouzari used sera specimens to obtain similar findings. The differences may be due to the limited sample size and specific age group in our study although using a urine sample for the detection of Anellovirus is rare. In the current study, as a preliminary one, we used urine samples for Anellovirus identification in Iran. Based on the phylogenetic tree, our introduced TTV isolates belonged to Alpha TTV species. It was found that 83.3% (5/6) of our Anelloviruses-positive isolates had significant similarity with TTV sequences from Germany, which could be a probable origin for circulating TTV strains in Iran.

Previous studies showed that the incidence rate of Anelloviridae increases with age. Anelloviridae has been detected in the feces of healthy male individuals over 81 years. Furthermore, a significant relationship has been observed between the presence of the virus in the feces of healthy people and the sex and age. TTV causes a chronic infection that could prolonge as permanent infection in the healthy host.[30,31] In our study, the higher frequency of infection was seen among females aged 6–8 years. It can be concluded that children with lower ages had less infection rates compared with other age groups. In the present study, the presence of viral infection was higher in female children, which has not been reported previously. Moreover, our study samples were urine, and the rate of viral infection in females' urine was higher than that of male participants. It has been suggested that Anelloviridae infection occurs in the 1st year of life.[32] In a follow-up study on healthy children under 10 years, TTMDV was isolated from 75% of the nasopharynx, 50% of feces, and 25% of urine specimens, and, interestingly, it was reported to be 46.7% in children with respiratory infections. It could be concluded that the presence of a virus may not be associated with respiratory tract illness.[33] In the present study, Beta and Gama TTVs were not detected and we also could not detect TTMV and TTMDV in the urine samples of healthy children. Moreover, a few studies in Iran have been performed on the prevalence of Anelloviridae in the healthy population. As for other countries, the prevalence of TTV viremia in healthy adults has been reported at 51.6% and 92% in Turkey and Japan, respectively.[34-36]

Our study limitations include the small size of the studied populations, the use of three primer–probe groups, specific age groups, i.e. children, asymptomatic participants, and using only urine samples, which possibly led to the existing controversies with other studies.

Conclusion

To the best of our knowledge, this is the first report of Anelloviridae detection in urine samples of asymptomatic people in Iran. Although we found 12% Anellovirus infection in our studied specimens, further studies are needed to investigate the blood and other organs of healthy people compared with immune-deficient ones. The impact of Anelloviridae infection on the immune response of healthy people can also be considered in future studies.

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Conflicts of interest

There are no conflicts of interest.

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