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Original Article

Optimization of RT-qPCR for Detection of Aichi Virus in Sewage and River Water Samples in Karaj, Iran

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Abstract

Background: Aichi virus (AiV) is an emerging virus, which belongs to *Kobuvirus* genus of the *Picornaviridae* family. AiV was recently determined as an etiologic agent of gastroenteritis in susceptible humans. After shedding of virus particles from affected people, AiV particles can contaminate water sources. Then, infection with this virus occurs in humans by the fecal-oral route after exposure with contaminated waters. Thus far, some research around the world demonstrated that different kinds of water sources including river water, ground water and treated or untreated sewage water have contamination with AiVs. Molecular detection of AiV has been mostly depended on reverse transcription polymerase chain reaction (RT-PCR) methods, which targeted 3CD junction region of the virus genome.

Methods: The present study aims to assess the molecular detection of AiVs in treated and untreated sewage water and river water specimens by the development of reverse transcription-quantitative PCR (RT-qPCR) assay for all AiV genotypes.

Results: Out of 50 samples tested (consisting of 28 river water samples and 22 sewage water samples), the AiV genomic RNA was identified in 15/28 (~50%) river water samples and in 14/22 (~70%) sewage samples.

Conclusion: Our results, for the first time, indicate that AiVs have been circulating in Iran.

Keywords: Aichi virus, Real-time PCR, Water

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Introduction

Many viruses were involved in enteric disease consisting of picornaviruses, rotaviruses, noroviruses, sapovirus, Astroviruses and adenoviruses.[1,2] However, there is a detection gap for samples in which the nonbacterial agent is not documented. Aichi virus (AiV) is an etiologic agent for enteric disease, which was first discovered from fecal sample of oyster-associated gastroenteritis during the outbreak in 1989 in Japan.[3] After then, the complete genome sequence of AiV was defined in 1998.[4] Since then, AiV species, including three genotypes A, B, and C, was located in the *Kobuvirus* genus in the *Picornaviridae* family.[3,5] Geographical distribution for each genotype has been determined; as genotype A has been found in Japan and Europe, but genotype B has been circulating in Brazil and other countries in Asia other than Japan and genotype C has been detected in Africa.[5,6] In addition to AiVs that can be infected humans, there are other kobuviruses, which are pathogens for animal species such as cattle, swine, dog, and mouse.[7,8] AiV particles are small-round naked viruses with one segment of single-stranded, positive-sense RNA genome.[4,5] The viral genome organization in length is 8,251 nucleotides (nts) and is the same as picornaviruses containing 5'NTR- leader protein- viral structural VP0, VP3 and VP1 proteins- nonstructural 2A, 2B, 2C, 3A, 3B,3C and 3D proteins- 3'NTR that end with a 3' poly (A) tail.[4,9] After the first report of isolation in Japan, AiVs have been detected by some studies during outbreaks of enteric illness with low incidence.[2,6,7,10-14] However, there are several seroprevalence studies that show a high AiV prevalence in adults (80% to 99%).[5,14-19]

On the other hand, environmental surveillance studies in different kinds of water sources have demonstrated that AiV presents in higher than 50% of contaminated water.[1,20,21] Thus, AiV is considered to be transmitted from contaminated water sources to human populations by oro-fecal transmission.[2,21,22] Also, it was found that the consumption of contaminated sea foods is responsible for this condition.[3,21,23] In general, surveillance studies onto sewage water and river water of studied areas are useful to assess AiV prevalence, their geographical genotype distribution, and health level. Based on comparisons of high seroprevalence detection of AiV antibodies with a low incidence of AiV genome detection, it is suggested that AiV infections frequently occur in asymptomatic individuals.[5,24,25] Finally, AiV infections which occur symptomatically are associated with clinical signs such as fever, diarrhea, abdominal pain, nausea

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and vomiting.[3,24] Virus isolation could be done by cell culture in BS-C 1 and Vero cells as described for the first time by Yamashita et al although its isolation is time-consuming.[3,21] Another methodology to detect AiVs is serology assay such as enzyme-linked immunosorbent assay (ELISA), but molecular methods including reverse transcription polymerase chain reaction (RT-PCR) and RT-quantitative PCR (RT-qPCR) have been introduced as a gold standard.[5,18,26,27] In addition, RT-PCR assay has been mostly used to detect AiV and to assess phylogenic genotypes with targeting of the 3CD junction region of their genomes.[5] However, RT-qPCR has recently been applied to the detection of RNA viruses in clinical and other samples. In the present study, two kinds of sample collected from sewage water (treated and untreated sewage water) and river water were evaluated to detect viral genomic RNA for all AiV genotypes by RT-qPCR using primers targeted to the 3CD junction region. Finally, we conducted a preliminary study to detect the AiVs in Iran.

Materials and Methods

Samples

During the cold season between September 2016 and May 2017, river water as well as treated and untreated samples were collected every week in Karaj city, Iran. A total of 22 sewage water samples and 28 river water samples in a volume of one liter were collected in sterile plastic bottles and shipped on ice and stored in −20°C. The samples were used for viral RNA extraction weekly after sample collection. Briefly, 100 ml of samples were centrifuged at 1500 ×g for 20 minutes, and the supernatant was collected for RT-PCR.

Viral RNA Extraction and Reverse Transcription

Viral genomic RNA was extracted from 100 µL of river and sewage water concentrated samples using the TRIZOL reagent according to the manufacturer's instruction. The viral RNA was eluted in a final volume of 50 µL. The concentration and quality of the extracted RNA were assessed by Nanovue spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNA was used directly in the reverse transcription reaction or stored at −70°C until use. Briefly, 10 µL of extracted RNA was added in RT mixtures containing random primer and incubate at 80°C for 10 minutes and after then placed on ice for 10 minutes. Then, second reaction buffer was added to the previous mixture and incubated at 42°C for 60 minutes. Finally, the RT reaction mixture incubates at 85°C for 5min to inactivate the enzyme. Nucleotide sequences of the partial 3CD region of AiV are highly conserved (99.2% to 97%), with amino acid identities of 99 to 100%, thus the selected previously used primers containing forward (5'-GACTTCCCCGGAGTCGTCGTCT-3') and reverse primers (5'-GCR GAGAATCCRCTCGTRCC-3') can amplify a 158-bp fragment located between the 3CD junction region of the AiV genotypes.[28]

Optimization of the qPCR Assay

The sequence of AiV genome located at 3CD junction region in length of 158 nt, were synthesized and cloned into pGH plasmid (Shanghai Generay Biotech Co.). A qPCR reaction using forward and reverse primers to detect all of AiV genotypes for each sample was performed in a 25 µL reaction volume containing 5 µL of synthesized cDNA or plasmid DNA (10^2 to 10^8 copies/reaction) as a template, 4 µL Syber master mix, and 10 mM of each primer. The negative control reaction also was included. PCR amplification was done with Rotorgen under the following program: primary denaturation at 95°C for 10 minutes, followed by 40 amplification cycles consisting denaturation at 95°C for 15 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 20 seconds. Amplification data were analyzed with Rotor-Gene Q software. All samples were characterized by a corresponding Ct value. Negative samples gave no Ct value.

Determination of Sensitivity and Specificity

The viral copy number was quantified through 10-fold serial dilution of plasmid DNA reactions. The slope (S) of the linear regression curve to access to efficiency and melting curve to determine PCR reaction quality were analyzed and results interpreted (Figure 1). For confirmation of results, five positive of Real-time PCR products were imaged by gel electrophoresis and purified. Then, sequencing was performed with the same primers used for amplification by using an ABI Prism Big Dye

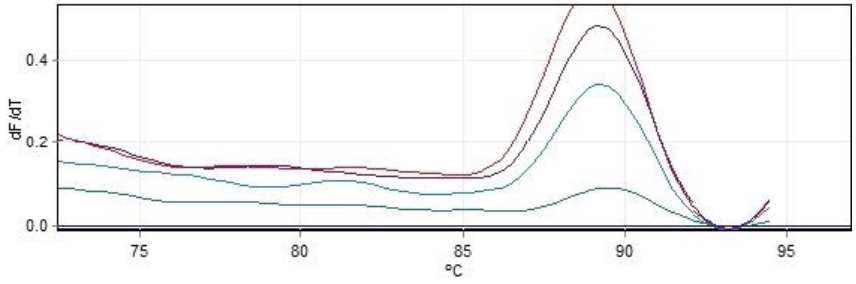

Figure 1. Melting Curves (*x* = temperature, *y* = changes in density of fluorescence signal) of the Samples. Each sample produced a single sharp peak, and all of them overlapped and showed the same melting temperature.

Terminator cycle sequencing.

Development of RT-Quantitative PCR

The standard curves were illustrated on the basis of the average cycle threshold (CT) values of reactions against to the amount of the plasmid copies per reaction volume.

The primer set was able to amplify plasmid DNA dilutions consist of 1.0×10^2 to 1.0×10^8 copies/reaction (Figure 2). The CT values were directly proportional to the log10 of the viral genome copies/reaction with correlation coefficients (r) of -0.99, and the slope of the standard curve was -3.2 (Figure 2). The lower quantification limit was determined to be around 1.0×10^2 copies per reaction. These results indicated that optimized primers to amplify 3CD junction region of AiV genotypes can be used for detection of the presence of low viral genomic RNA in environmental including river water and sewage water samples. Also, the optimized RT-qPCR has a capacity to detect AiV viral genomic RNA in clinical samples.

Detection of AiV Genomic RNA in River Water

To assess the applicability of the RT-qPCR assay for detection of AiV in river water samples, we tested a total of 28 samples. Out of 28 samples for detection of AiVs genome, the developed RT-qPCR illustrated positive signals for 15 samples (53.57%). According to the standard curve, the concentration of viral genomic RNA was seen from range 3.4×10^2 to 5.9×10^6 copies/liter of river water sample.

Detection of AiV Genomic RNA in Treated and Untreated Sewage Water

This assay was also implemented in order to determine the presence of AiV in sewage water samples including treated and untreated samples. Out of 22 collected samples consisting 12 treated and 10 untreated sewage water for detection of AiVs RNA genome, the RT-qPCR was positive for 7 samples of treated sewage samples (58.33%) and positive for 7 samples of untreated sewage water samples (70%). The viral RNA load in treated and untreated sewage samples were shown to be range from 4.2×10^3 to 6.7×10^5 copies/liter and 2.1×10^4 to 1.9×10^6 copies/liter, respectively.

Discussion

After the first identification of AiV by Yamashita et al in Japan, many studies have determined AiV prevalence among children and elderly peoples as well as in environmental polluted samples including river water, treated and untreated water.[3,5] The VP1 region has been generally used to divide the *Picornaviridae* family into genera based on its nucleotide sequence diversification features, but the 3CD junction region has been shown to be sufficient for AiV genotype analysis.[4,10,21] In this study, we implemented RT-qPCR methods for detection and quantification of AiV in river water as well as treated and untreated water samples. One set of primer targeting the conserved 3CD junction region, which was previously used for all AiV genotypes detection,[28] was used to amplify AiVs by the optimized RT-qPCR assay. In general, the present study is the first detection of AiV in Iran.

To date, some studies have conducted RT-qPCR assay to detect AiVs and to measure AiV RNA load in clinical and environmental samples.[5,25] Here, fifty samples collected from river water as well as sewage water were subjected to the RT-qPCR assay. According to previous data, which demonstrated higher prevalence of AiV in cold seasons, our tested samples were collected from September to May. Many studies from around the world have illustrated AiV prevalence in water specimens with loads ranging from low to moderate and high percentage.[1,20-23,29] In the present study, the rate of detection for AiVs in river water was around 53.57 percent and in treated and untreated sewage water samples were 58.33 and 70 percent, respectively. Our results were coincident with previous Tunisian, Netherlands and Japanese studies, which reported 50% up to 100% of the wastewater samples were contaminated with AiVs.[1,20,21]

After isolation of AiV from oyster-associated gastroenteritis in 1989, several environmental surveillance studies have suggested the possible transmission route of AiV from contaminated water sources to humans.[3,5] Thus, the first occurrence of AiVs in Iran could be partly caused by a gap in explanation for the unknown viral etiologic agent to induce enteric illness. Regarding previously published documents, which demonstrated extended distribution of AiV, here we explained the occurrence of

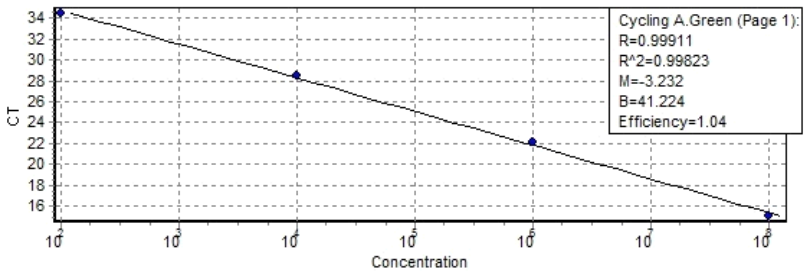

Figure 2. Sensitivity of Aichi Virus Detection by Rotorgen Real-Time PCR in a Series of 10-Fold Dilutions of Genomic AiV– Plasmid (10^2 to 10^8) Per PCR Reaction.

AiV in Alborz province may be result in occurrence of the AiV in other locations and also in people with diarrhea that remains to be determined.[5,6,11,14,15] Regarding a previous study implemented in Japan, which detected AiVs with 2.3% prevalence among Pakistani children samples with gastroenteritis, it seems that presence of AiV in Iran can potentially infect people especially children with or without gastroenteritis.[11]

The AiV RNA detection in both treated (58.33%) and untreated sewage water (70%) could be explained by the treatment processes of sewage water which are not efficient to remove of AiV particles because of possible virus aggregation forms or crystalline formation groups, as such has been previously described.[21,30] On the other hand, the higher viral load in treated and untreated sewage water samples were similar to previous work, which showed a high AiV detection in cold seasons.[25]

Finally, the occurrence of AiVs in both river water and sewage water because of virus shedding by feces of involved peoples increase the necessity of more environmental water monitoring to reduce the risk of virus transmission to humans.

In conclusion, through RT-qPCR, the AiV was detected in environmental samples. Further studies are necessary to implement the optimized RT-qPCR for analysis of clinical samples. The viral load can be appraised by this method.

Authors' Contribution

ZA performed this project as part of her MSc, MG designed and managed the project, and all the other authors were involved in the experiment, technical support and writing of the manuscript.

Conflict of Interest Disclosures

The authors have no conflicts of interest.

Ethical Statement

None to be declared.

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