Aichivirus in Children with Diarrhea in Northern Italy

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Keywords

Aichivirus A · Real-time polymerase chain reaction · Gastroenteritis · RNA extraction · Coinfection

Abstract

Objective: Since its discovery, Aichivirus (AiV) A has been detected, with an incidence of 0.9–4.1%, primarily when studying outbreaks of diarrhea in children or young adults. In this paper, we report the first detection of AiV in Piedmont, Italy, in pediatric patients. **Methods:** A total of 159 fecal specimens (from 96 males and 63 females) previously screened for rotaviruses, adenoviruses, noroviruses, human parechoviruses, saliviruses, and sapoviruses were collected from infants and children with acute gastroenteritis. **Results:** The most commonly detected virus was norovirus GII (33.80%), followed by rotavirus (21.30%), astrovirus (18.87%), bocavirus (13.92%), sapovirus (10.90%), parechovirus (8%), norovirus GI (6.70%), adenovirus (1%), and salivirus (0.52%). Real-time polymerase chain reaction detected AiV A in 1 (0.62%) case subjects. AiV A was detected in monoinfection only in January. **Conclusions:** Our results indicate that AiV may be associated with a limited number of diarrhea cases in pediatric patients.

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Introduction

Gastroenteritis is a common disease in children, characterized by diarrhea, vomiting, abdominal pain, and fever. Pediatric gastroenteritis remains the second most common cause of child mortality worldwide [1]. Diarrheal disease can be caused by a wide spectrum of pathogens (parasites, bacteria, and viruses). In the last decade, there has been a great shift from conventional methods to identify viral pathogens to the utilization of polymerase chain reaction (PCR) assays, which have proven their advantages over previously used techniques such as rapid antigen detection and viral culture [2]. The major benefits of molecular diagnostic methods are lower detection limits and, therefore, a higher analytical sensitivity for common viruses such as rotaviruses (RV) and adenoviruses (AdV), and their ability to detect uncultivable viruses such as noroviruses (NoV), sapoviruses (SaV), and astroviruses (HAstrV) [3–6]. Moreover, real-time PCR has the ability to determine the viral load in a semiquantitative way, expressed as the amount of amplification cycles (cycle threshold value) necessary to detect the pathogen. There is, however, a large diagnostic gap, resulting in the etiologic agent not being identified in approximately a

third of all specimens, and it has been suggested that other viruses, including picornaviruses, are involved [7]. Aichi virus (AiV) was first discovered in 1989 during a search for an infectious agent in an outbreak of oyster associated nonbacterial gastroenteritis [8]. The isolated virus was later classified as the first member of a novel genus, i.e., *Kobuvirus*, of the family Picornaviridae [9]. Kobuviruses have since then been associated with diarrhea not only in humans but also in cattle [10, 11] and swine [12, 13]. Since its discovery, AiV has been detected with an incidence of 0.9–4.1%, primarily when studying outbreaks of diarrhea in children or young adults [14–18]. AiV seems to be circulating worldwide, and detection of AiV has been reported in Asia [19], South America [20], Europe [13–15, 20], and Africa [17]. Previous studies have suggested oysters, or other seafood, to be the source of AiV infections [2, 14, 17, 21, 22] but its occurrence has also been reported in sewage [17, 23], which indicates that kobuviruses might use the fecal-oral route of infection, like several other picornaviruses. Although doubts exist regarding its clinical role because of frequent codetection (50–80%) of AiV and other viral agents [14, 15, 24] and low incidence rates (0.9–4.1%) in acute gastroenteritis [15, 24–26], other studies have recently yielded contrasting results, showing that codetection of AiV with other common enteropathogenic viruses occurs at a low rate [27] and that this virus is rarely detected in healthy individuals [27–29], which supports a possible role of this virus in gastroenteritis. In this paper, we report the first detection of AiV in Piedmont, Italy. In addition, this is the first paper describing the presence of AiV in Piedmont pediatric patients.

Materials and Methods

Target Sequence Selection

The primers and probe were designed based on alignment of all of the available relevant virus sequences in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) in July 2010 using ClustalW (<http://www.clustal.org/>) and Primer3 software (<http://frodo.wi.mit.edu/primer3/>). The primers and probe target a highly conserved part of the 5' untranslated region [26]. The primers for internal control, i.e., glyceraldehyde-3-phosphate dehydrogenase (GAPDH), have been previously published [30].

Fecal Specimens

A total of 159 fecal specimens (from 96 males and 63 females) previously screened for NoV, RV, and AdV (192 samples), human parechovirus (HPeV) and SaV (137 samples), and SalV, HAstrV, and human bocavirus (HBoV) (123 samples) were collected from infants and children with acute gastroenteritis in the Pediatric Hospital Regina Margherita (Turin in Italy) from December 2014 to November 2015 and tested for human AiV. This

study was performed according to the guidelines of the institutional review board on the use of biologic specimens for scientific purpose in keeping with Italian law (art. 13 D.Lgs 196/2003). The samples were collected from children under the age of 5 years with acute gastroenteritis in the Pediatric Hospital Regina Margherita from December 2014 to April 2015. Acute gastroenteritis was defined as the rapid onset of 2 or more of the following symptoms: diarrhea, vomiting, nausea, fever, and abdominal pain. Patients who met any of the following criteria were not eligible for this study: immunosuppressive therapy and age >5 years. This study was approved by our institutional ethics committee on November 24, 2014 (protocol No. 116918).

Nucleic Acid Extraction

A total of 50 µg of fecal specimens (previously diluted 1/10 in 0.9% NaCl buffer saline solution) were extracted on a Maxwell automated extractor (Promega, Madison, WI, USA) using the RNA Blood Kit protocol without modifications. Samples were eluted in 50 µL of distilled water.

Reverse Transcription

For reverse transcription (RT), 5 µL of the stored, extracted RNA was added to a reagent mixture consisting of 5× first-strand buffer (Applied Biosystems, Carlsbad, CA, USA), 10 mM dNTP (Sigma, St. Louis, MO, USA), MuLV (50 U/µL) (Applied Biosystems), a random primer (50 µM, hexa-deoxyribonucleotide mixture; Promega), RNase inhibitor (40 U/µL) (Promega), and distilled water. The total volume of the reaction mixture was 50 µL. The RT reaction was carried out at 42 °C for 15 min, followed by 99 °C for 5 min and then 5 °C for 5 min [30].

Real-Time PCR

Optimization of the PCR reaction was performed by determining the optimal amount of primers and probe, as well as the cycling conditions. The optimized AiV PCR reaction contained 5 µL of extracted DNA, 200 nM of AiV primer forward, 900 nM of AiV primer reverse, 200 nM of the AiV FAMprobe, and 1× PCR Master Mix (Applied Biosystems) in a total of 20 µL of reaction. The PCR amplification was carried out for 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C, and 1 min at 60 °C on a 7500 ABI real-time instrument (Applied Biosystems).

Internal Control

AiV and GAPDH PCR amplification were performed in singleplex reactions to evaluate the potential interference between PCR targets. A negative AiV result was considered valid only if the internal control GAPDH probe showed amplification.

Gastroenteric Virus Detection

The following information was extracted from the medical record database: age, gender, RV and AdV. HPeV, SaV, NoV, SalV, HAstrV, and HBoV were detected as previously reported [31–33].

Specificity

The analytical specificity of the RT PCR assay was determined by testing coxsackievirus B1 (ATCC VR-28), B2 (ATCC VR-29), and B3 (ATCC VR-30), enterovirus type 68 (ATCC VR-561), 69 (ATCC VR-785), 70 (ATCC VR-836), and 71 (ATCC VR-1432), human orthopneumovirus (ATCC VR-26), influenza viruses (influenza A virus H1N1 [ATCC VR-95], H3N2 [ATCC VR-547], and influenza B virus [ATCC VR-101]), herpes simplex virus 1 (ATCC VR-260), human betaherpesvirus 5 (ATCC VR-538), hu-

Fig. 1. Monthly distribution of the major viral pathogens in pediatric patients with gastroenteritis.

Table 1. Detected enteric viruses: coinfection data

Viral pathogen	Infected stools	Coinfection
Norovirus GI	13/192	7/13 (53.8)
Norovirus GII	65/192	30/65 (46.1)
Rotavirus	41/192	19/41 (46.3)
Adenovirus	2/192	1/2 (50)
Parechovirus	11/137	10/11 (90.9)
Sapovirus	15/137	10/15 (66.6)
Bocavirus	17/123	13/17 (76.47)

Values are presented as numbers/total or numbers/total (%).

man gammaherpesvirus 4 (ATCC VR-602), human coronavirus types 229E (ATCC VR-740) and OC43 (ATCC VR-1558), *Escherichia coli* (ATCC 25922), *Legionella pneumophila* (ATCC 33152), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 14579), *Haemophilus influenzae* (ATCC 49766), *Staphylococcus aureus* (ATCC 25923), and *Lactobacillus plantarum* (ATCC 8014).

Results

Common Viral Agents in Children

The mean age of the tested patients was 21.6 ± 10.3 months. The majority of the children (86%) were 1–36 months of age. The most commonly detected virus was NoV GII (33.80%), followed by RV (21.30%), HAstV

(18.87%), HBoV (13.92%), SaV (10.90%), HPeV (8%), NoV GI (6.70%), AdV (1%), and SaV (0.52%). The monthly distribution of the viral pathogens is described in Figure 1, which suggests that gastrointestinal virus detection peaked during the Italian winters; however, since the number of tested stools was rather limited, a larger sampling will be required to confirm any seasonality pattern. Multiple infections with 1–4 viruses responsible for gastroenteritis in humans stool were found (Table 1).

Detection of AiV

Real-time PCR detected AiV in 1 (0.62%) subject. The virus was detected in a viral monoinfection in January.

Discussion

Currently, RT PCR is used widely and considered to be a convenient, useful, and powerful method for molecular diagnosis to detect pathogens from clinical specimens. With the development of real-time PCR assays, many of the limitations related to performing PCR amplification in a clinical laboratory have been overcome. In the present study, stool specimens ($n = 159$) obtained from Italian children aged <5 years who presented with acute diarrhea were retrospectively analyzed for AiV using RT real-time PCR. The stool specimens were collected from 2014 to 2015 in the city of Turin (Italy). Since its

discovery, AiV has been detected with an incidence of 0.9–4.1%, primarily when studying outbreaks of diarrhea in children or young adults [14–18]. Our results confirmed that enteric viruses play an important role in pediatric diarrhea, and further that the most common viral agent associated with gastroenteritis is NoV, followed by RV, HAstrV, HBoV, SaV, HPeV, AdV, and SalV. The prevalence rates of RV, SaV, HPeV, AdV, and HBoV were similar to those reported previously [32, 34–36]. However, the detection rates of 40.5 and 18.87% for NoV and HAstrV, respectively, in this study were higher than those reported previously in Italy [37–39] but similar to those in the recent study of Rovida et al. [40]. Moreover, RV detection peaked during spring, which is in conflict with the results of previous studies [41].

AiV has been associated with gastroenteritis in children and young adults in Germany [20], France [14], Hungary [13], Tunisia [17], Pakistan [18], China [27], and Finland [15]. Our data showed that the detection rate of AiV in diarrheal children (0.62%) was similar to those observed in other countries [15, 26, 42–45]. A limitation of our study is the small number of stool samples from gastroenteritis and the lack of samples from healthy controls. In conclusion, the present study investigated the presence of AiV in pediatric patients with diarrhea in Piedmont, Italy. Our results indicate that AiV may be associated with a limited number of diarrhea cases even in elderly pediatric patients. The discrepancies between the

high seroprevalence and the low incidence of detection could be due to AiV infections being mainly asymptomatic. Seroprevalence studies have shown that 80–95% of the population in their 30s has antibodies against AiV [20, 46, 47], which indicates a general and frequent circulation of the virus among humans. The reported incidence of AiV is in general low, but it is significantly higher in outbreaks of gastroenteritis associated with shellfish [14, 17]. However, the high seroprevalence, even in the very young population, indicates the possibility of other transmission routes. AiV has recently been detected in sewage and sewage-polluted water in Venezuela [23] and Tunisia [17], which indicates a circulation of AiV in the environment. Given the low prevalence of AiV in our population, the AiV result will require confirmation using a much larger sample size from healthy individuals. AiV remains rare, and the significance of AiV as a human pathogen is a question that remains to be further investigated.

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Disclosure Statement

The authors have no conflict of interests to declare.

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