Journal of Clinical Virology 87 (2017) 37–42

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Analysis of Aichi virus and Saffold virus association with pediatric acute gastroenteritis

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ARTICLE INFO

Article history:
Received 1 November 2016
Received in revised form 2 December 2016
Accepted 9 December 2016

Keywords:
Saffold virus
Aichi virus
Pathogenicity
Real-time PCR
Case-control

ABSTRACT

Background: Aichi virus (AiV) and Saffold virus (SAFV) have been reported in children with acute gastroenteritis and respiratory disease worldwide; however, their causative role in acute gastroenteritis remains ambiguous.
Objectives: To assess the clinical association of AiV and SAFV with acute gastroenteritis in the pediatric population.
Study design: A case-control study involving 461 paired stool samples from pediatric cases with diarrhea and healthy controls was conducted in China. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was used to screen AiV and SAFV.
Results: In the 461 paired samples, AiV and SAFV were more prevalent among asymptomatic children than children with acute gastroenteritis (0.87% vs. 0.43% and 2.8% vs. 1.5%, respectively), with no significant differences between groups ($p = 0.142$ and $p = 0.478$, respectively). Cox regression model analysis revealed no correlation between AiV (odds ratio, OR = 2.24; 95% confidence interval, CI, 0.76–6.54) or SAFV infection (OR = 1.36; 95% CI, 0.86–2.15) and diarrhea. High viral loads were found in both AiV- and SAFV-positive groups, with no significant difference in viral load between the groups ($p = 0.507$ and $p = 0.677$, respectively). No other known enteric pathogens were found in the AiV-positive samples but common in SAFV-positive cases. Phylogenetic analysis revealed that all 6 AiV subjects clustered with genotype B. All 7 SAFV-positive cases and 8 of 13 SAFV-positive controls were genotyped successfully; the genotypes identified included SAFV-1, SAFV-2 SAFV-3, and SAFV-6.
Conclusion: Our study revealed no association of these viruses in acute gastroenteritis in children. These viruses may have the ability to replicate in humans; however, the infections are usually asymptomatic.

1. Background

Many novel viruses have been discovered in recent years as a result of advances in next generation sequencing (NGS) and other highly sensitive molecular detection technologies. Currently, at least 54 species of *Picornaviridae* grouped into 30 genera (http://www.picornaviridae.com/) have been identified, and representatives of seven of these genera have been found in humans (*Enterovirus, Hepatovirus, Parechovirus, Cardiovirus, Salivirus, Cosavirus,* and *Kobuvirus*) [1].

Aichi virus (AiV), belonging to the genus *Kobuvirus* of the family *Picornaviridae*, was first isolated from a 1989 fecal sample of a patient in Japan with acute gastroenteritis, which was likely caused by consumption of an oyster [2]. AiV can be classified into three genotypes with different distributions [3], and epidemiologic studies indicate a global circulation of AiV in various human populations [3–10]. Both AiV genotypes A and B have been reported in Japan [11], Finland [8], South Korea [3], and Thailand [6]. Genotype A was reported in Sweden [12], Hungary, France [5], and Tunisia [14], whereas Genotype B was identified in Germany [7] and China [15]. Nevertheless, the role that AiV plays in gastroenteritis remains unclear [3,5,7]. Most previous studies assessing the prevalence of AiV in children with gastroenteritis have lacked sufficiently large samples and quantitative data regarding viral load. Furthermore, few case-control studies have addressed this topic.

Saffold viruses (SAFV), belonging to genus *Cardiovirus* of the family *Picornaviridae*, was first identified in 2007 from the stool of an infant in 1981 with a fever of unknown origin [16]. SAFV exhibits high genetic diversity and has been found in cases with acute flac-

Abbreviations: AiV, Aichi virus; SAFV, Saffold virus.

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http://dx.doi.org/10.1016/j.jcv.2016.12.003
1386-6532/

cid paralysis [17], respiratory tract infections [18], and diarrhea in China [19–21]. Currently, eleven genetic lineages have been identified, and SAFV-1, SAFV-2, and SAFV-3 are distributed globally. The prevalence rate of SAFV ranges from 0.5 to 3.2% in patients with diarrhea [18–23]. Although several studies [19,22,23] have investigated the clinical relevance of SAFV with acute gastroenteritis or respiratory tract diseases, correlative studies have been hampered due to coinfections with other known diarrhea-causing viruses or the lack of healthy controls [7,18,19,23].

2. Objectives

Our aim was to assess the clinical association of AiV and SAFV with acute gastroenteritis in a case-control study using real-time polymerase chain reaction (PCR), and investigated the genotype prevalence of both viruses in China.

3. Study design

3.1. Study participants and sample collection

We conducted a matched case-control study with hospitalized children with diarrhea and healthy children in northern China (Lulong, Hebei province) and southern China (Liuyang, Hunan province) [24] from May 2011 to January 2013. Case patients were children younger than 5 years of age with acute diarrhea. Case subjects were defined as those having three or more loose, liquid, or watery stools or at least one bloody loose stool passed in 24 h, and their parents reported clinical symptoms (fever, vomiting, dehydration) and demographic information in a questionnaire. Healthy controls were enrolled at the same sites within 14 days of enrollment of the matched cases. Healthy controls were defined as those having no diarrhea or vomiting symptom within one week, and they were matched with the cases according to sex and age. A total of 461 case-control pairs were enrolled: 239 pairs were from northern China (Lulong, Hebei province) and 222 pairs were from southern China (Liuyang, Hunan province).

3.2. Nucleic acid amplification and detection

Viral RNA samples were extracted from 200 μl of 10% fecal suspension in phosphate-buffered saline using the QIAamp MiniElute Virus spin kit (Qiagen), according to the manufacturer's instructions.

All specimens were quantified for SAFV and AiV by real-time reverse transcription (RT) PCR using the AgPath-ID One-Step RT-PCR kit (Applied Biosystems, Foster City, CA) and virus-specific primers and probes as described previously [7,23].

All positive samples were typed via nested RT-PCR and sequencing. For AiV, the 266-bp 3CD junction region was amplified by nested PCR using the 6261/6779 and C94b/246k primer sets as described previously [11]. SAFV was detected using two sets of nested PCR primers: the CardioVP1-1F/4R and CardioVP1-2F/3R primer sets [18], and VP1F1/VP1R1 and VP1F2/VP1R2 primer sets targeting the VP1 region as described previously [25]. The PCR products were separated on 1.5% agarose gels, and the amplicons of appropriate sizes were purified using the QIA quick gel extraction kit (Qiagen), followed by sequencing. Quantitative PCR was used to analyze stool samples for 10 other known diarrhoeal pathogens (Rotavirus, norovirus GII, norovirus GI, sapovirus, astrovirus, adenovirus, Salmonella, Shigella, *Campylobacter jejuni*, and ETEC) [24].

3.3. Phylogenetic analysis

Nucleotide sequence alignments were performed using ClustalW. Phylogenetic evolutionary analyses were conducted using MEGA (v5.05), and trees were generated using the neighbor-joining algorithm method that included 1000 bootstrap replicates.

3.4. Statistical analysis

Chi-squared statistical analysis was performed to determine the difference in proportions of samples that were positive or negative by quantitative PCR. Independent two-tailed *t*-tests were used to compare the difference in virus copy number in cases with diarrhea and healthy controls. The matched case and control groups were compared using Cox regression analysis to determine the association between virus infections and acute gastroenteritis.

4. Results

4.1. Prevalence of AiV and SAFV

Of the 461 pairs of stool samples from case and control groups, AiV was identified in 2 (0.43%) patients (age range, 12–18 months) with diarrhea and in 4 (0.87%) healthy children (age range, 1–55 months). Therefore, AiV was more prevalent among asymptomatic children than children with diarrhea, although the prevalence rate was low in both groups. χ^2-tests revealed no significant differences in AiV infection between the groups ($p = 0.142$). We found no coinfections with additional diarrhea pathogens such as rotavirus, norovirus, astrovirus, adenovirus, shigella, salmonella, *Campylobacter jejuni*, and *E. coli* in the 2 AiV-positive cases (data not shown).

SAFV was detected in 7 (1.5%) diarrhea cases and 13 (2.8%) healthy controls; the χ^2-test revealed no significant difference in SAFV infection between the groups ($p = 0.478$). Coinfection with other known diarrhea pathogens including rotavirus, norovirus, astrovirus adenovirus, shigella, salmonella, *Campylobacter jejuni* and *E. coli* were common in the SAFV-positive samples (data not shown); 5 of 7 SAFV-positive samples from patients revealed coinfection: 1 with rotavirus, 3 with norovirus GII, 1 with sapovirus, and 1 with adenovirus. One of these samples revealed triple-infection with rotavirus and adenovirus. In addation, 3 of 13 SAFV-positive controls had co-infection with other known diarrhea pathogens.

Independent two-tailed *t*-tests revealed no significant differences in age, sex, or geographical location between the case and control groups for AiV and SAFV-positive subjects.

4.2. Quantitative analysis of AiV and SAFV

We used real-time PCR to quantify the AiV and SAFV load. High RNA copy numbers of up to 2.8×10^7 per g of stool were found in diarrhea cases (Table 1). However, the viral loads in these two groups did not differ significantly ($p = 0.507$). Two AiV positive-cases occurred in July and August 2012, and both originated from a geographically restricted area within Hunan province.

The viral loads of SAFV in the case group ranged from 6.8×10^2 to 1.3×10^6 per g (median = 1.3×10^5), while those in the control group ranged from 4.3×10^2 to 4.7×10^6 per g (median = 5.3×10^3) (Table 1); however, *t*-test analysis revealed no significant difference in viral load between the two groups ($p = 0.677$).

4.3. Association of AiV or SAFV with diarrhea

In this study population, AiV and SAFV were more prevalent among asymptomatic children than children with gastroenteritis.