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EPIDEMIOLOGY OF AICHI VIRUS IN FECAL SAMPLES FROM OUTPATIENTS WITH ACUTE GASTROENTERITIS IN NORTHWESTHERN SPAIN.

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ABSTRACT

Background: In recent years, Aichi virus (AiV) has been involved in acute viral gastroenteritis outbreaks. However, the common pathogenesis of AiV releases more in subclinical infections underestimating the impact of AiV in human health.

Objectives: The present study describes the presence and genetic diversity of AiV in patients with gastroenteritis in Northwestern Spain.

Study design: A total of 2,667 stool samples, obtained between July 2010 and June 2011, from diarrheic outpatients were studied for detection and molecular characterization of AiV using PCR techniques followed by sequencing and phylogenetic analyses.

Results: The virus was detected in 124 (5.0%) of the samples among all age groups. Coinfections were also detected, from the 124 positive samples, 72 (58.1%) were positive only for AiV, whereas mixed contaminations with *Norovirus* genogroup I or genogroup II, *Sapovirus*, or other enteric pathogens were detected in 52 (41.9%) samples. A total of 70 positive samples could be genotyped, being characterized as genotype A (58.6%) or B (41.4 %). AiV was detected from August to April, being the highest number of AiV positive samples detected during autumn and winter seasons.

Conclusions: This survey remarks the importance of emerging enteric viruses in patients who require medical assistance, and offers more information about the real importance of AiV as gastroenteritis agent.

Keywords: Aichi virus, genotyping, epidemiology, gastroenteritis.

Highlights

- Prevalence of Aichi virus was determined in outpatients from NW Spain
- The virus was detected in 5.0% of the samples among all age groups
- Monoinfections (58.1%) were more abundant than mixed infections (41.9%)
- Genotypes A and B were detected in all age groups.

1. Background

Viral gastroenteritis constitutes a common human illness, which continues to be a significant cause of morbidity and mortality worldwide [1,2]. *Norovirus* (NoV), *Rotavirus* (RV), *Adenovirus* (AdV) and *Astrovirus* are considered the most important aetiological agents of acute non-bacterial gastroenteritis outbreaks [3,4]. In recent years, human Aichi virus (AiV) has also been involved in acute viral gastroenteritis outbreaks [5].

AiV was first recognized in 1989 from a case of gastroenteritis associated with oyster consumption in Japan [6]. Clinical symptoms include diarrhoea, abdominal pain, nausea, vomiting and fever, but the common pathogenesis of AiV releases more in subclinical infections than in clinically manifest diseases [7,8]. This fact underestimates the real impact of AiV in human health and explains why many studies demonstrated a high prevalence of AiV antibodies in adults (80%-99%), indicating a great exposure to AiV, but a low incidence of AiV in clinical samples from sporadic or epidemic gastroenteritis outbreaks [9].

AiV is a virus with icosahedral morphology that presents a positive-sense single-stranded RNA genome. AiV belongs to the genus *Kobuvirus* within the family *Picornaviridae*, and consists in six recently renamed species: *Aichivirus A*, *Aichivirus B*, *Aichivirus C*, *Aichivirus D*, *Aichivirus E* and *Aichivirus F* [10,11]. *Aichivirus A* is divided in six genetically distinct groups: AiV [7], canine kobuvirus [12], murine kobuvirus [13], Kathmandu sewage kobuvirus [14], roller kobuvirus [15], and feline kobuvirus [16].

Genetically, AiV has been divided in a single serotype and three genotypes: AiV A, common in Europe, Asia, and Africa [17–20]; AiV B, detected in America, Asia, and

Europe [17,21]; and AiV C that was found in a child hospitalized in France that had returned from a trip to Africa [18,22].

Real-time reverse transcription-quantitative PCR (RT-qPCR) assay is a widely used method for AiV identification because it is a rapid and sensitive tool for specific detection and quantitative analysis [23], helpful to determine the circulation of the virus among human populations. Also, conventional RT-PCR coupled with amplicon sequencing has been used for the detection and genotyping of AiV by targeting the viral protein 1 (VP1) [24]. This protein is genetically diverse and useful to establish a timeline for the emergence of AiV variants in different geographic regions.

2. Objectives

Previous studies carried out in our laboratory demonstrated the presence of AiV in Galician molluscs [25]. In this work, a total of 2,667 stool samples from outpatients with acute gastroenteritis in Galicia (Northwestern Spain) were studied for detection and characterization of AiV with the aim to determine its prevalence and predominant genotypes in this region.

3. Study design

3.1. Stool Samples

Stool samples included in this study were obtained from Complejo Hospitalario Universitario de A Coruña, Galicia (NW Spain), which serves more than 550,000 people in an area of 2,750 km^2. A total of 2,667 specimens from outpatients of all ages affected with gastroenteritis were collected during a 1-year period (July 2010–June 2011). For subsequent data analysis, six different age-groups were established: 0–2 years (886 samples), 3–5 years (195 samples), 6–12 years (244 samples), 13–18 years

(71 samples), 19–59 years (653 samples), and >60 years (597 samples). Also, twenty-one samples with unknown age were included.

3.2. Viral stocks

AiV strain A846/88 [7] was kindly provided by Dr. Javier Buesa (University of Valencia, Spain). Mengovirus clone (vMC0) was kindly provided by Dr Albert Bosch (University of Barcelona, Spain).

3.3. Viral recovery and RNA extraction

Viral recovery from original stool samples was carried out as previously described [25]. Briefly, known amounts of Mengovirus clone vMC0 were spiked into each sample homogenate (10 ul, 10^3 PFU) for RNA extraction efficiency control [26]. Supernatants (150 µl) recovered after homogenization in peptone water and centrifugation, were utilized for viral RNA extraction using Nucleospin®RNA Virus Kit (Macherey-Nagel, Düren, Germany). The RNA was eluted in RNase-free sterile water and stored at –80°C.

3.4. RT-qPCR detection and quantification

Viral RNA (5 µl) was tested using Platinum® Quantitative RT-PCR ThermoscriptTM One-step System kit (Invitrogen; France) in a 25 µl total volume, Negative controls containing no nucleic acid as well as positive controls were introduced in each run. The RT-qPCR for AiV was performed on an Mx3005p QPCR System (Stratagene; USA) thermocycler. Extraction and amplification efficiencies were calculated according to the ISO 15216-1:2017 specifications [27] using Mengovirus and appropriate external controls [26,28].

Amplification conditions for AiV were reverse transcription at 45°C for 10 min, denaturation at 95°C for 10 min, followed by 40 cycles of amplification with annealing at 95°C for 15 s and extension at 45°C for 60 s, using the primers described by Kitajima et al. [29] (Table 1). Quantification was carried out following the principles outlined in the ISO 15216-1:2017 [27] as previously described [25].

3.5. AiV genotyping

Viral RNA of all positive samples was subjected for genotyping using a RT-nested PCR protocol designed by Lodder et al. [24] (Table 1). Amplicons of the expected length were purified and directly sequenced at STABVida Lda. (Portugal). Sequences obtained were processed with Lasergene 7 software package (DNASTAR Inc., Madison, WI) and aligned using MEGA version 6 software package [30]. Phylogenetic tree was built by the maximum-likelihood method (bootstrap of 1,000 replicates). Sequences of AiV reference strains were obtained from GenBank. Sequences of AiV strains detected in the present study are available at GenBank under accession numbers LS479128 to LS479168 and LS481153 to LS481181. AiV sequences obtained from shellfish samples (GenBank accession numbers LS97418 to LS974201) in Galician estuaries [25] were also included in the phylogenetic tree with comparative purposes.

3.6. Statistical analyses

Pearson's chi-squared tests were performed to evaluate differences among AiV prevalences in the different age-groups, as well as to determine correlations among genotypes and age-groups. Analyses were carried out using IBM$^{®}$ SPSS$^{®}$ Statistics 20 software (IBM Corp., USA).

4. Results

4.1. AiV prevalence

All stool samples showed acceptable RNA extraction (>5%) and RT-qPCR (>25 %) efficiencies. AiV were detected in 124 (4.7%) of the total samples, being present in patients of all age. The highest prevalence was observed in children between 3-5 years, (5.6%), followed by patients between 19-59 years (4.9%). In infants under 2 years, AiV were observed in 4.9% of the patients. Other age groups showed lower prevalences, 4.2% in teenagers between 13-18 years old, 4.1% in children between 6-12 years old, and 3.7% in people older than 60 years (Figure 1). No significant statistical differences ($p>0.05$) were detected for the AiV prevalences among the several age-groups.

4.2. Mono and mixed infections

The AiV detection was comparatively analyzed with results previously obtained in our laboratory in the same stool samples [31,32], in order to detect coinfections of AiV with other enteric pathogens.

Just over half of the positive samples (58.1%) appeared as AiV monoinfections (Figure 2). The 0-2 years, 19-59 years and >60 years were the age groups where most of these monoinfections were detected (Supplementary Table 1). Only in the age group 13-18 years all the positive samples constituted monoinfections of AiV, but it is also noteworthy that it was the age-group with a lowest number of samples analyzed (Supplementary Table 1).

Mixed infections comprised a variety of enteric pathogens, including viruses, bacteria and parasites (Figure 2; Supplementary Table 1). Among these, the more abundant were coinfections with other viruses. Coinfections of AiV and *Sapovirus* (SaV) were detected in 11 samples, followed by coinfections of AiV with NoV genogroup I (GI) (7 samples) or NoV genogroup II (GII) (7 samples). Three viral types were detected in 10 samples (5 with AiV, NoV GI and SaV, and 5 with AiV, NoV GII and SaV). A random distribution of these mixed infections among the different age groups was observed, although in general were more abundant in groups 0-2 years, 3-5 years and 19-59 years (Supplementary Table 1). No coinfections by AiV, NoV GI and GII were detected.

Co-infections only with bacterial pathogens were detected in 9 of the AiV positive samples (Figure 2; Supplementary Table 1). Most cases of these mixed infections were with *Campylobacter* spp. (4 samples), followed by *Salmonella* spp. (3 samples) and *Aeromonas* spp. (2 samples), being generally more abundant in patients under 2 years. One sample in the group 0-2 years rendered positive for AiV and the parasite *Cryptosporidium* (Figure 2; Supplementary Table 1).

In a total of 7 samples, the presence of three or four bacterial and viral agents was detected (Figure 2; Supplementary Table 1). Thus, triple infections of AiV, SaV and *Campylobacter*, AiV, SaV and *Yersinia enterocolitica* or AiV, NoV GII and *Yersinia enterocolitica* were observed in patients of 0-2 years. Multiple infections by AiV, NoV GI, SaV and *Campylobacter* were detected in two samples, one in the group 0-2 years and the other in the group 3-5 years. Other polyinfections included AiV, NoV GI, SaV and *Bacillus cereus,* detected in a sample from the group 19-59 years, and AiV, NoV GII, SaV and *Campylobacter*, detected in a sample from the group 6-12 years (Figure 2; Supplementary Table 1).

4.3. AiV quantification

Quantification levels for AiV in feces ranged from 7.14 x 10^2 GC/g, detected in a clinical sample from the age group of >60 years old, to 6.38 x 10^8 GC/g, detected in a stool sample from a child in the age group of 0-2 years with a mixed infection of AiV, NoV GII and SaV. The mean values of AiV were also estimated by age group, ranging from 9.81 x 10^3 GC/g obtained for the age group of ≥60, to 4.12 x 10^4GC/g obtained for the age group of 0-2 years (Table 2).

4.4. Epidemiology

Seasonally, AiV was detected from August 2010 to April 2011, although the highest number of positive samples was observed during autumn and winter seasons (Figure 3). October was the month with more positive samples followed by January, September and February, 85.4% of the positive samples being detected in these 4 months.

Seventy out of 124 positive samples could be genotyped. Forty-one (58.6%) samples belonged to genotype A and 29 (41.4 %) samples to genotype B (Table 3; Figure 4). Genotype C was not observed in this study. Most of genotype A samples were related to strain A846/88, originally isolated from an oyster-associated gastroenteritis outbreak in Japan (Figure 4). Genotype A and B sequences were present in samples from all groups. The genotype A was especially abundant in infants under 2 years (22.9%) while genotype B was more prevalent in adults between 19-59 years (20%) (Table 3), being such correlations statistically significant ($p<0.05$). Finally, the 4 molluscan samples included were classified as genotype B and two of them were phylogenetically related to clinical samples with similarities higher than 97.5% (Figure 4).

5. Discussion

Aichi virus has emerged in the last years as a gastroenteritis agent of considerable importance in different parts of the world, as evidenced by different seroprevalence studies [17,18,33,34]. Isolation of the virus from patients with gastroenteritis was reported in Japan [35], Southeast Asia [19,21], Germany and Brazil [17]. In addition, AiV RNA was detected in stool samples from some other European countries, including France, Hungary or Finland [18,36,37]. The present study constitutes the first survey on prevalence of AiV in Spain using molecular methods for direct detection of viral RNA.

AiV prevalence (≈ 5%) observed in the present study, although at levels sligthly higher, is in agreement with the low incidence reported in other surveys from other geographic regions [19,22,38]. As previously described, this low prevalence contrasts with the results obtained from seroepidemiological studies [17,35,39], which indicate that practically all the population with age >50 show antibodies to AiV. Specifically in

Spain, a high seroprevalence (>85%) was observed for people over 20 years old, suggesting a general exposition to this human pathogen in our country [33] probably through subclinical infections.

Our results show no great differences among the different age groups, all of them with prevalences around 4-5%. These results are in contrast with those reported from Germany [17], where most of infections seem to occur among children younger than 6 years. Most of studies on AiV prevalence have been focussed on children cohorts [18,20,35,36]. Prevalences obtained in the present study for the age groups 0-2 years (4.85%) and 3-5 years (5.64%) are higher than those observed in France [18], Hungary [37] or Finland [36], and at comparable levels to those observed in Tunisia [20].

It has been suggested that the presence of AiV in samples from gastroenteritis outbreaks could be considered as an indicator of mixed infections [18]. However, the results obtained here, rendering a high percentage of monoinfections (58.1%), seem to support the hypothesis of Sdiri-Loulizi et al. [34] who suggested the role of AiV as a real pathogenic agent virulent enough to cause the need of medical care and/or hospitalization.

Certain geographical distribution of the AiV genotypes can be deduced from the literature. Thus, genotype A is predominant in Japan and has also been detected in Germany and France [17–19]. Genotype B is predominant in Bangladesh, and has been observed in Brazil, Pakistan, Malaysia, and Nigeria [17,19,21,40]. Similar prevalence of these two genotypes was reported in Finland [36], although the number of positive samples was too low to obtain firm conclusions. On the other hand, genotype C was detected once in France from one patient returning from a trip to Africa [18].

In the present study, genotypes A and B were detected, showing genotype A sligthly higher prevalence. It is interesting to point out that genotype A was more prevalent in infants of 0-2 years, whereas genotype B was more abundant in the age group 19-59 years. On the other hand, the AiV detected in molluscan samples in the same geographic area were characterized as genotype B, showing high similarity with some clinical samples. Such results may indicate that contaminated shellfish may constitute a via of transmission of AiV within human population in this area, and that feed habits may be responsible in part of the genotype-drift from genotype A to B with the age. Taking togeteher all this data, the replacement of viral types over the age groups could be explained by variations in transmission of AiV by direct contact, food, or travelling [41], although further studies are needed to gain more knowledge on the genotype distribution worldwide.

Although AiV was present along the year, different monthly prevalences were observed being the majority of positive samples concentrated in autumn and winter months. Factors like climatological and oceanographic conditions, international travelling, or seasonality of mollusc consumption could influence to these seasonal peaks [42].

In summary, recent molecular methods for screening and characterization of gastroenteritis pathogens revealed novel enteric virus as the cause of diarrhoeal illness and outbreaks. The present study confirms the presence of AiV in gastroenteritis patients, offering more information about the importance of this virus on human health in our region. However, it is important to point out that taking together these results and those from previous works in our group, the aetiology could only be established for aproximately 50% of all the gastroenteritis cases studied. This fact suggests that other enteric pathogens may be present and further studies are needed in order to identify them and to determine their real public health importance.

Conflict of interest

The authors declare no conflict of interest.

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Author's contributions

JLR designed the work. ER and MFV performed the experiments. ER and JLR analyzed the data. ER and JLR wrote the paper. All authors revised the manuscript, read and approved the final draft. JLR supervised the study.

Ethical approval

This study was carried out in accordance with the Declaration of Helsinki as revised in 2000. This non-interventional study included no additional procedures. Anonymized biological material was obtained only for standard viral diagnosis. The Spanish Biomedical Research law (14/2007; article 3i) does not require written informed consent for such a protocol.

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Figure legends

Figure 1. Human AiV prevalence within the different age-groups.

Figure 2. Total number of AiV cases in coinfection with other viral and bacteriological agents. Data for comparison obtained from Manso and Romalde [31] and Varela et al. [32].

Figure 3. Distribution of the AiV prevalence along the period of study.

Figure 4. Phylogenetic tree of AiV samples based on VP1 region sequences by neighbor-joining analysis using MEGA 6. Galician samples are shown in bold type. Asterisks indicate sequences obtained from shellfish samples. Bootstrap values (greater than 50%) are shown at each node as percentages of 1,000 replicates. GenBank accession numbers are detailed in the tree. Bar, nucleotide substitutions per site.

1. Background

Viral gastroenteritis constitutes a common human illness, which continues to be a significant cause of morbidity and mortality worldwide [1,2]. *Norovirus* (NoV), *Rotavirus* (RV), *Adenovirus* (AdV) and *Astrovirus* are considered the most important aetiological agents of acute non-bacterial gastroenteritis outbreaks [3,4]. In recent years, human Aichi virus (AiV) has also been involved in acute viral gastroenteritis outbreaks [5].

AiV was first recognized in 1989 from a case of gastroenteritis associated with oyster consumption in Japan [6]. Clinical symptoms include diarrhoea, abdominal pain, nausea, vomiting and fever, but the common pathogenesis of AiV releases more in subclinical infections than in clinically manifest diseases [7,8]. This fact underestimates the real impact of AiV in human health and explains why many studies demonstrated a high prevalence of AiV antibodies in adults (80%-99%), indicating a great exposure to AiV, but a low incidence of AiV in clinical samples from sporadic or epidemic gastroenteritis outbreaks [9].

AiV is a virus with icosahedral morphology that presents a positive-sense single-stranded RNA genome. AiV belongs to the genus *Kobuvirus* within the family *Picornaviridae*, and consists in six recently renamed species: *Aichivirus A*, *Aichivirus B*, *Aichivirus C*, *Aichivirus D*, *Aichivirus E* and *Aichivirus F* [10,11]. *Aichivirus A* is divided in six genetically distinct groups: AiV [7], canine kobuvirus [12], murine kobuvirus [13], Kathmandu sewage kobuvirus [14], roller kobuvirus [15], and feline kobuvirus [16].

Genetically, AiV has been divided in a single serotype and three genotypes: AiV A, common in Europe, Asia, and Africa [17–20]; AiV B, detected in America, Asia, and

Europe [17,21]; and AiV C that was found in a child hospitalized in France that had returned from a trip to Africa [18,22].

Real-time reverse transcription-quantitative PCR (RT-qPCR) assay is a widely used method for AiV identification because it is a rapid and sensitive tool for specific detection and quantitative analysis [23], helpful to determine the circulation of the virus among human populations. Also, conventional RT-PCR coupled with amplicon sequencing has been used for the detection and genotyping of AiV by targeting the viral protein 1 (VP1) [24]. This protein is genetically diverse and useful to establish a timeline for the emergence of AiV variants in different geographic regions.

2. Objectives

Previous studies carried out in our laboratory demonstrated the presence of AiV in Galician molluscs [25]. In this work, a total of 2,667 stool samples from outpatients with acute gastroenteritis in Galicia (Northwestern Spain) were studied for detection and characterization of AiV with the aim to determine its prevalence and predominant genotypes in this region.

3. Study design

3.1. Stool Samples

Stool samples included in this study were obtained from Complejo Hospitalario Universitario de A Coruña, Galicia (NW Spain), which serves more than 550,000 people in an area of 2,750 km^2. A total of 2,667 specimens from outpatients of all ages affected with gastroenteritis were collected during a 1-year period (July 2010–June 2011). For subsequent data analysis, six different age-groups were established: 0–2 years (886 samples), 3–5 years (195 samples), 6–12 years (244 samples), 13–18 years

(71 samples), 19–59 years (653 samples), and >60 years (597 samples). Also, twenty-one samples with unknown age were included.

3.2. Viral stocks

AiV strain A846/88 [7] was kindly provided by Dr. Javier Buesa (University of Valencia, Spain). Mengovirus clone (vMC0) was kindly provided by Dr Albert Bosch (University of Barcelona, Spain).

3.3. Viral recovery and RNA extraction

Viral recovery from original stool samples was carried out as previously described [25]. Briefly, known amounts of Mengovirus clone vMC0 were spiked into each sample homogenate (10 ul, 10^3 PFU) for RNA extraction efficiency control [26]. Supernatants (150 µl) recovered after homogenization in peptone water and centrifugation, were utilized for viral RNA extraction using Nucleospin®RNA Virus Kit (Macherey-Nagel, Düren, Germany). The RNA was eluted in RNase-free sterile water and stored at –80°C.

3.4. RT-qPCR detection and quantification

Viral RNA (5 µl) was tested using Platinum® Quantitative RT-PCR ThermoscriptTM One-step System kit (Invitrogen; France) in a 25 µl total volume, Negative controls containing no nucleic acid as well as positive controls were introduced in each run. The RT-qPCR for AiV was performed on an Mx3005p QPCR System (Stratagene; USA) thermocycler. Extraction and amplification efficiencies were calculated according to the ISO 15216-1:2017 specifications [27] using Mengovirus and appropriate external controls [26,28].

Amplification conditions for AiV were reverse transcription at 45°C for 10 min, denaturation at 95°C for 10 min, followed by 40 cycles of amplification with annealing at 95°C for 15 s and extension at 45°C for 60 s, using the primers described by Kitajima et al. [29] (Table 1). Quantification was carried out following the principles outlined in the ISO 15216-1:2017 [27] as previously described [25].

3.5. AiV genotyping

Viral RNA of all positive samples was subjected for genotyping using a RT-nested PCR protocol designed by Lodder et al. [24] (Table 1). Amplicons of the expected length were purified and directly sequenced at STABVida Lda. (Portugal). Sequences obtained were processed with Lasergene 7 software package (DNASTAR Inc., Madison, WI) and aligned using MEGA version 6 software package [30]. Phylogenetic tree was built by the maximum-likelihood method (bootstrap of 1,000 replicates). Sequences of AiV reference strains were obtained from GenBank. Sequences of AiV strains detected in the present study are available at GenBank under accession numbers LS479128 to LS479168 and LS481153 to LS481181. AiV sequences obtained from shellfish samples (GenBank accession numbers LS97418 to LS974201) in Galician estuaries [25] were also included in the phylogenetic tree with comparative purposes.

3.6. Statistical analyses

Pearson's chi-squared tests were performed to evaluate differences among AiV prevalences in the different age-groups, as well as to determine correlations among genotypes and age-groups. Analyses were carried out using IBM® SPSS® Statistics 20 software (IBM Corp., USA).

4. Results

4.1. AiV prevalence

All stool samples showed acceptable RNA extraction (>5%) and RT-qPCR (>25 %) efficiencies. AiV were detected in 124 (4.7%) of the total samples, being present in patients of all age. The highest prevalence was observed in children between 3-5 years, (5.6%), followed by patients between 19-59 years (4.9%). In infants under 2 years, AiV were observed in 4.9% of the patients. Other age groups showed lower prevalences, 4.2% in teenagers between 13-18 years old, 4.1% in children between 6-12 years old, and 3.7% in people older than 60 years (Figure 1). No significant statistical differences ($p>0.05$) were detected for the AiV prevalences among the several age-groups.

4.2. Mono and mixed infections

The AiV detection was comparatively analyzed with results previously obtained in our laboratory in the same stool samples [31,32], in order to detect coinfections of AiV with other enteric pathogens.

Just over half of the positive samples (58.1%) appeared as AiV monoinfections (Figure 2). The 0-2 years, 19-59 years and >60 years were the age groups where most of these monoinfections were detected (Supplementary Table 1). Only in the age group 13-18 years all the positive samples constituted monoinfections of AiV, but it is also noteworthy that it was the age-group with a lowest number of samples analyzed (Supplementary Table 1).

Mixed infections comprised a variety of enteric pathogens, including viruses, bacteria and parasites (Figure 2; Supplementary Table 1). Among these, the more abundant were coinfections with other viruses. Coinfections of AiV and *Sapovirus* (SaV) were detected in 11 samples, followed by coinfections of AiV with NoV genogroup I (GI) (7 samples) or NoV genogroup II (GII) (7 samples). Three viral types were detected in 10 samples (5 with AiV, NoV GI and SaV, and 5 with AiV, NoV GII and SaV). A random distribution of these mixed infections among the different age groups was observed, although in general were more abundant in groups 0-2 years, 3-5 years and 19-59 years (Supplementary Table 1). No coinfections by AiV, NoV GI and GII were detected.

Co-infections only with bacterial pathogens were detected in 9 of the AiV positive samples (Figure 2; Supplementary Table 1). Most cases of these mixed infections were with *Campylobacter* spp. (4 samples), followed by *Salmonella* spp. (3 samples) and *Aeromonas* spp. (2 samples), being generally more abundant in patients under 2 years. One sample in the group 0-2 years rendered positive for AiV and the parasite *Cryptosporidium* (Figure 2; Supplementary Table 1).

In a total of 7 samples, the presence of three or four bacterial and viral agents was detected (Figure 2; Supplementary Table 1). Thus, triple infections of AiV, SaV and *Campylobacter*, AiV, SaV and *Yersinia enterocolitica* or AiV, NoV GII and *Yersinia enterocolitica* were observed in patients of 0-2 years. Multiple infections by AiV, NoV GI, SaV and *Campylobacter* were detected in two samples, one in the group 0-2 years and the other in the group 3-5 years. Other polyinfections included AiV, NoV GI, SaV and *Bacillus cereus*, detected in a sample from the group 19-59 years, and AiV, NoV GII, SaV and *Campylobacter*, detected in a sample from the group 6-12 years (Figure 2; Supplementary Table 1).

4.3. AiV quantification

Quantification levels for AiV in feces ranged from 7.14 x 10^2 GC/g, detected in a clinical sample from the age group of >60 years old, to 6.38 x 10^8 GC/g, detected in a stool sample from a child in the age group of 0-2 years with a mixed infection of AiV, NoV GII and SaV. The mean values of AiV were also estimated by age group, ranging from 9.81 x 10^3 GC/g obtained for the age group of ≥60, to 4.12 x 10^4GC/g obtained for the age group of 0-2 years (Table 2).

4.4. Epidemiology

Seasonally, AiV was detected from August 2010 to April 2011, although the highest number of positive samples was observed during autumn and winter seasons (Figure 3). October was the month with more positive samples followed by January, September and February, 85.4% of the positive samples being detected in these 4 months. Seventy out of 124 positive samples could be genotyped. Forty one (58.6%) samples belonged to genotype A and 29 (41.4 %) samples to genotype B (Table 3; Figure 4). Genotype C was not observed in this study. Most genotype A samples were related to strain A846/88, originally isolated from an oyster-associated gastroenteritis outbreak in Japan (Figure 4). Genotype A and B sequences were present in samples from all groups. The genotype A was especially abundant in infants under 2 years (22.9%) while genotype B was more prevalent in adults between 19-59 years (20%) (Table 3), being such correlations statistically significant ($p<0.05$). Finally, the 4 molluscan samples included were classified as genotype B and two of them were phylogenetically related to clinical samples with similarities higher than 97.5% (Figure 4).

5. Discussion

Aichi virus has emerged in the last years as a gastroenteritis agent of considerable importance in different parts of the world, as evidenced by different seroprevalence studies [17,18,33,34]. Isolation of the virus from patients with gastroenteritis was reported in Japan [35], Southeast Asia [19,21], Germany and Brazil [17]. In addition, AiV RNA was detected in stool samples from some other European countries, including France, Hungary or Finland [18,36,37]. The present study constitutes the first survey on prevalence of AiV in Spain using molecular methods for direct detection of viral RNA. AiV prevalence (≈ 5%) observed in the present study, although at levels sligthly higher, is in agreement with the low incidence reported in other surveys from other geographic regions [19,22,38]. As previously described, this low prevalence contrasts with the results obtained from seroepidemiological studies [17,35,39], which indicate that practically all the population with age >50 show antibodies to AiV. Specifically in

Spain, a high seroprevalence (>85%) was observed for people over 20 years old, suggesting a general exposition to this human pathogen in our country [33] probably through subclinical infections.

Our results show practically no differences among the different age groups, all of them with prevalences around 4-5%. These results are in contrast with those reported from Germany [17], where most of infections seem to occur among children younger than 6 years. Most of studies on AiV prevalence have been focussed on children cohorts [18,20,35,36]. Prevalences obtained in the present study for the age groups 0-2 years (4.9%) and 3-5 years (5.6%) are higher than those observed in France [18], Hungary [37] or Finland [36], and at comparable levels to those observed in Tunisia [20].

It has been suggested that the presence of AiV in samples from gastroenteritis outbreaks could be considered as an indicator of mixed infections [18]. However, the results obtained here, rendering a high percentage of monoinfections (58.1%), seem to support the hypothesis of Sdiri-Loulizi et al. [34] who suggested the role of AiV as a real pathogenic agent virulent enough to cause the need of medical care and/or hospitalization.

Certain geographical distribution of the AiV genotypes can be deduced from the literature. Thus, genotype A is predominant in Japan and has also been detected in Germany and France [17–19]. Genotype B is predominant in Bangladesh, and has been observed in Brazil, Pakistan, Malaysia, and Nigeria [17,19,21,40]. Similar prevalence of these two genotypes was reported in Finland [36], although the number of positive samples was too low to obtain firm conclusions. On the other hand, genotype C was detected once in France from one patient returning from a trip to Africa [18].

In the present study, genotypes A and B were detected, showing genotype A sligthly higher prevalence. It is interesting to point out that genotype A was more prevalent in infants of 0-2 years, whereas genotype B was more abundant in the age group 19-59 years. On the other hand, the AiV detected in molluscan samples in the same geographic area were characterized as genotype B, showing high similarity with some clinical samples. Such results may indicate that contaminated shellfish may constitute a via of transmission of AiV within human population in this area, and that feed habits may be responsible in part of the genotype-drift from genotype A to B with the age. Taking togeteher all this data, the replacement of viral types over the age groups could be explained by variations in transmission of AiV by direct contact, food, or travelling [41], although further studies are needed to gain more knowledge on the genotype distribution worldwide.

Although AiV was present along the year, different monthly prevalences were observed being the majority of positive samples concentrated in autumn and winter months. Factors like climatological and oceanographic conditions, international travelling, or seasonality of mollusc consumption could influence to these seasonal peaks [42].

In summary, recent molecular methods for screening and characterization of gastroenteritis pathogens revealed novel enteric virus as the cause of diarrhoeal illness and outbreaks. The present study confirms the presence of AiV in gastroenteritis patients, offering more information about the importance of this virus on human health in our region. However, it is important to point out that taking together these results and those from previous works in our group, the aetiology could only be established for aproximately 50% of all the gastroenteritis cases studied. This fact suggests that other enteric pathogens may be present and further studies are needed in order to identify them and to determine their real public health importance.

Conflict of interest

The authors declare no conflict of interest.

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Author’s contributions

JLR designed the work. ER and MFV performed the experiments. ER and JLR analyzed the data. ER and JLR wrote the paper. All authors revised the manuscript, read and approved the final draft. JLR supervised the study.

Ethical approval

This study was carried out in accordance with the Declaration of Helsinki as revised in 2000. This non-interventional study included no additional procedures. Anonymized biological material was obtained only for standard viral diagnosis. The Spanish Biomedical Research law (14/2007; article 3i) does not require written informed consent for such a protocol.

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Figure legends

Figure 1. Human AiV prevalence within the different age-groups.

Figure 2. Total number of AiV cases in coinfection with other viral and bacteriological agents. Data for comparison obtained from Manso and Romalde [31] and Varela et al. [32].

Figure 3. Distribution of the AiV prevalence along the period of study.

Figure 4. Phylogenetic tree of AiV samples based on VP1 region sequences by neighbor-joining analysis using MEGA 6. Galician samples are shown in bold type. Asterisks indicate sequences obtained from shellfish samples. Bootstrap values (greater than 50%) are shown at each node as percentages of 1,000 replicates. GenBank accession numbers are detailed in the tree. Bar, nucleotide substitutions per site.

Tables 1-3

Table 1. Primers and probe employed in the study for AiV detection and sequencing

Primers and Probes	Sequence (5´-3´)[a]	Nucleotide location	Reference
RT-qPCR Primers			[29]
AiV-AB-F	GTCTCCACHGACACYAAYTGGAC	1882–1904[b]	
AiV-AB-R	GTTGTACATRGCAGCCCAGG	1970–1989[b]	
RT-qPCR Probe			[29]
AiV-AB-TP	FAM-TTYTCCTTYGTGCGTGC-MGB	1939–1955[b]	
RT-Nested PCR primers			[24]
AiV-VP3-F1	CACACCGCCCCTGCGTCRGCCCTCGT	2912–2937[c]	
AiV-VP1-F2	CTCGATGCRCCMCAAGACACCGG	3023–3045[c]	
AiV-VP1-F3	GTGCTTCACRTACATCGCYGCGG	3289–3311[c]	
AiV-VP1-R2	CCTGACCAGTCCTCCCAWCCGAAGTA	3552–3527[c]	
AiV-VP1-R1	GAGAGCTGGAAGTCRAAGGG	3651–3632[c]	

[a] Mixed bases in degenerate primers and probe are as follows: H represents A, C, or T; R represents A ; Y represents C or T; M indicates A or C and W indicates A or T.

[b] Position of the AiV primers is of the 5´base relative to AiV reference strain no. AB040749.

[c] Position of the AiV primers is of the 5´base relative to AiV reference strain no. AB010145.

Table 2. Quantification of AiV levels (log genome copies/g feces) by age group.

Age group (yr.)	**Mean**	**SD**	**Range**
0-2	4.61	8.00	3.00-8.80
3-5	4.56	6.70	3.31-7.22
6-12	4.46	7.71	3.54-8.21
13-18	4.15	4.21	3.80-4.57
19-59	4.36	7.58	3.34-8.34
≥60	3.99	6.01	2.85-6.67

Table 3. Genotype of sequenced samples by age group

Age group	**Genotype A**		**Genotype B**	
	No. of positives	**%**	**No. of positives**	**%**
0-2 yr	16	22.9	6	8.6
3-5 yr	3	4.3	4	5.7
6-12 yr	4	5.7	1	1.4
13-18 yr	2	2.9	0	0.0
19-59 yr	9	12.9	14	20.0
> 60 yr	7	10.0	4	13.8
Total	41	58.6	29	41.4

Figure 1

Click here to download high resolution image

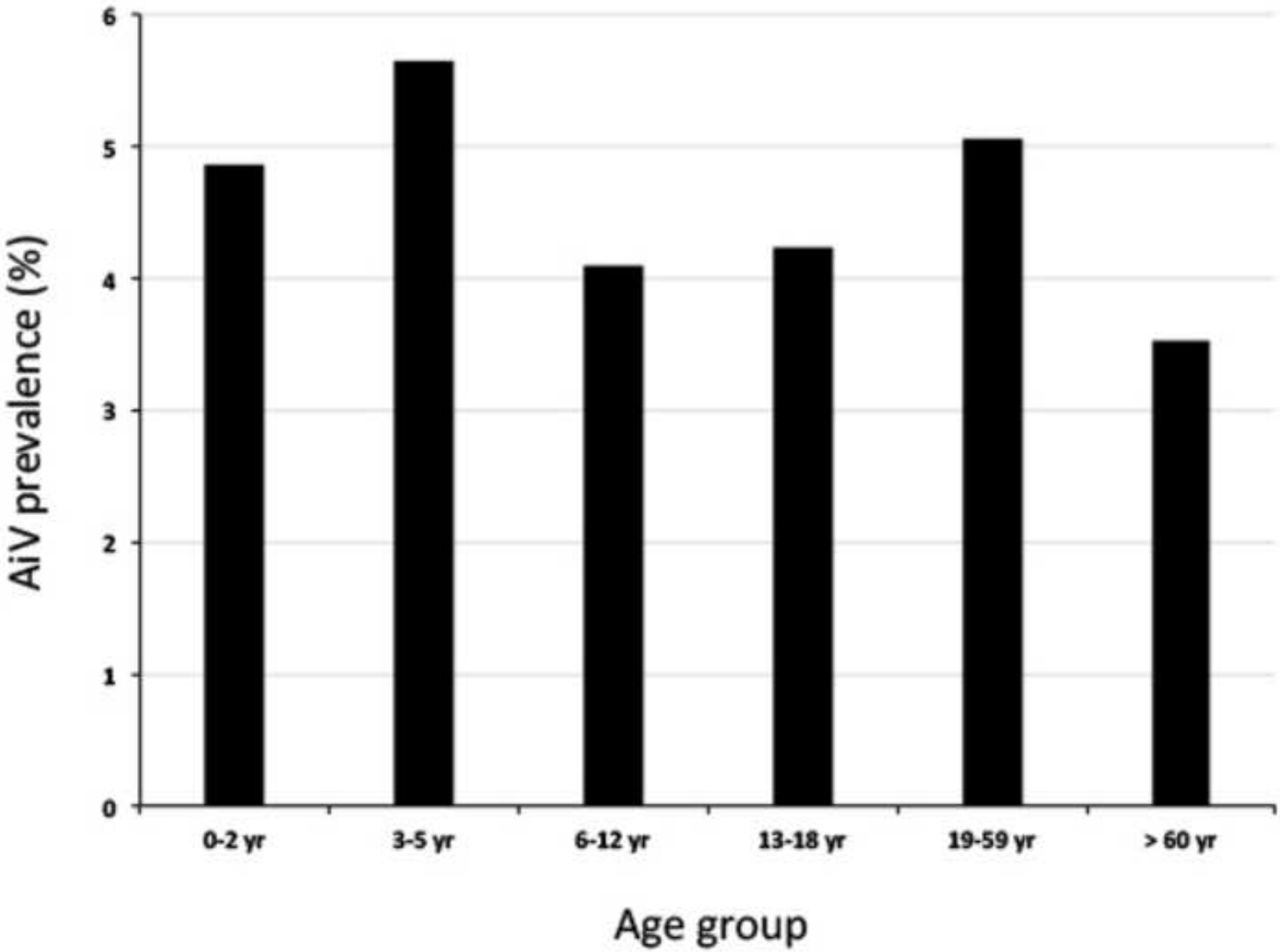


Figure 2

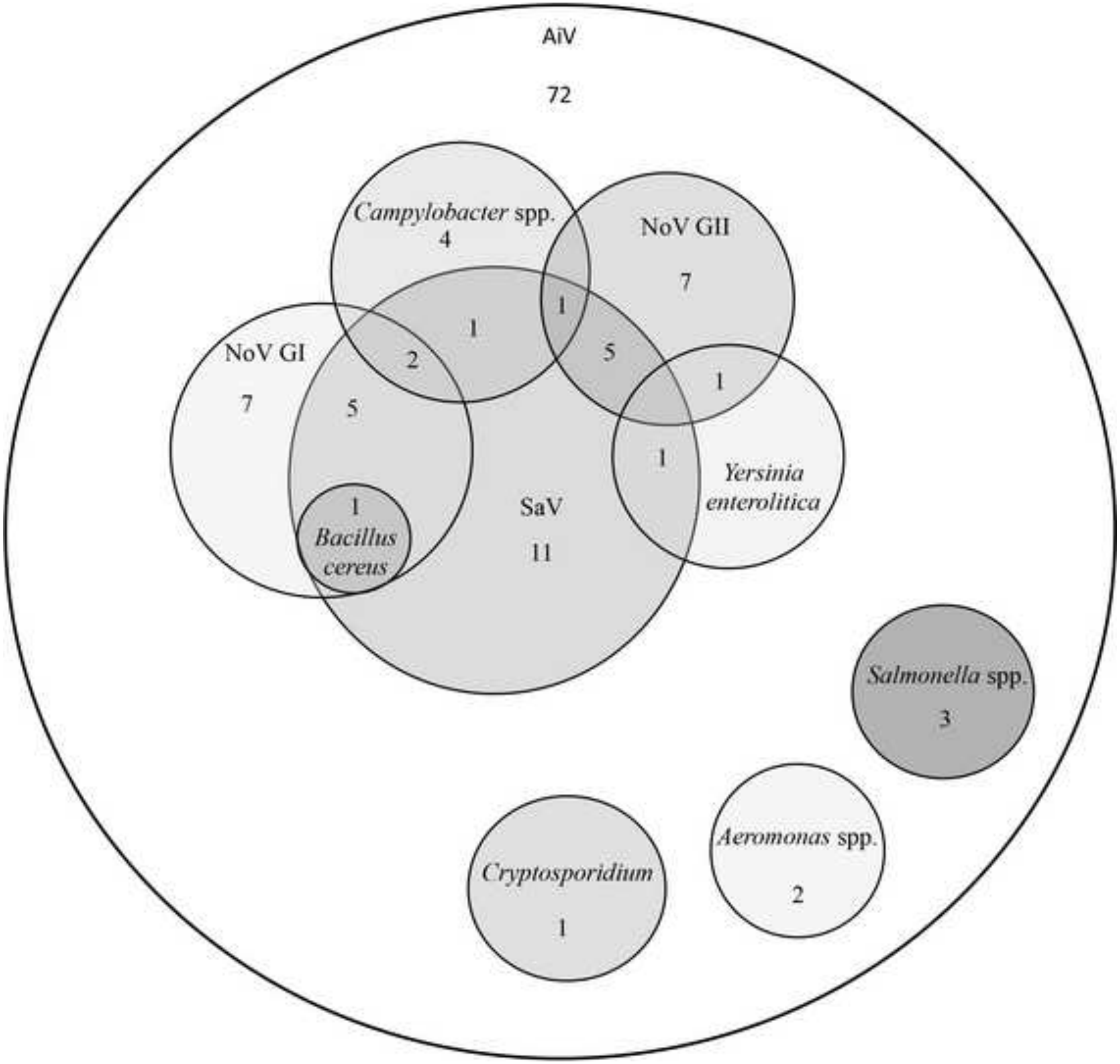

AiV
72
Campylobacter spp.
4
NoV GII
7
1
1
NoV GI
2
7
5
5
1
1
1
Bacillus cereus
SaV
11
Yersinia enterolitica
Salmonella spp.
3
Aeromonas spp.
2
Cryptosporidium
1

Figure 3

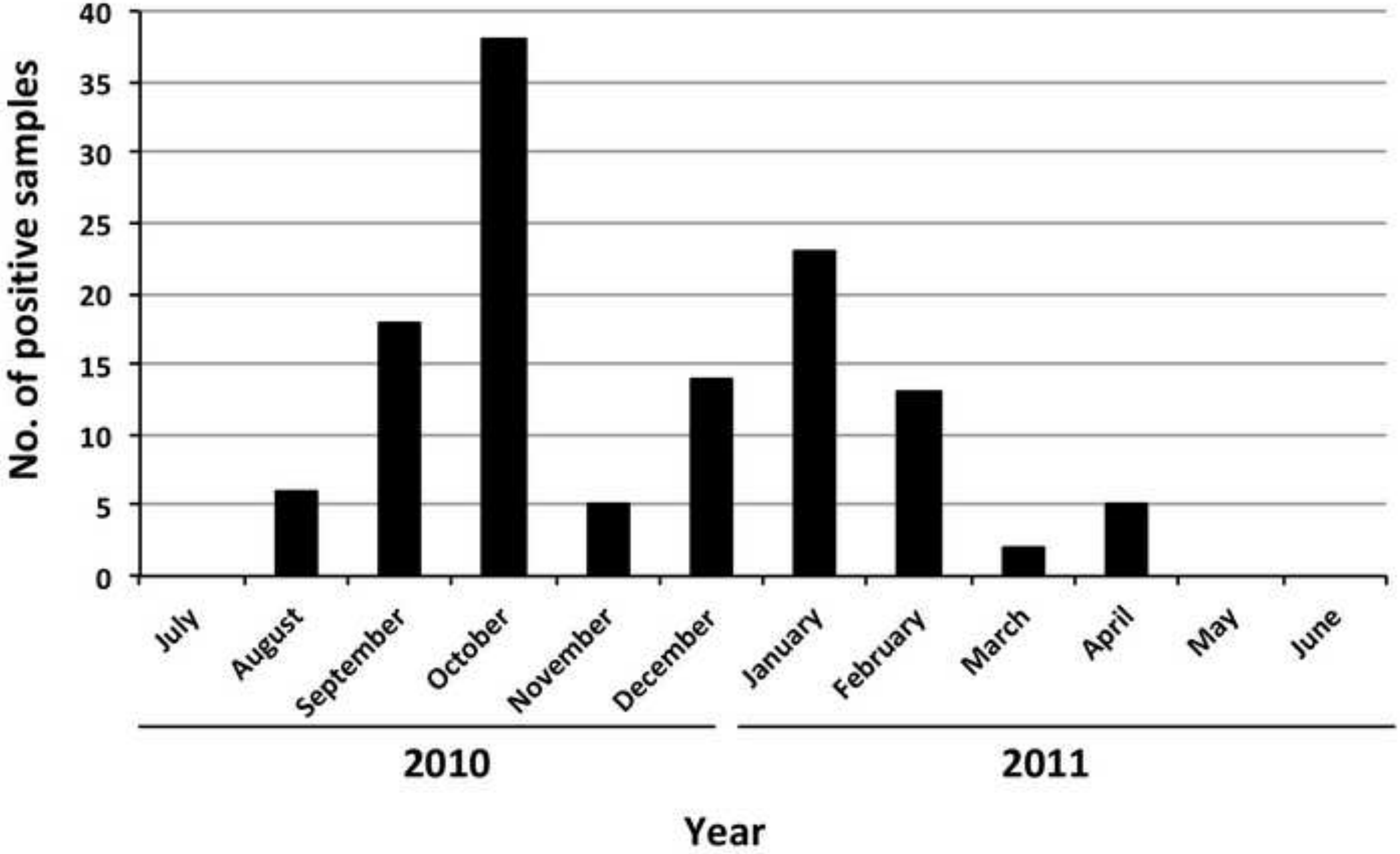
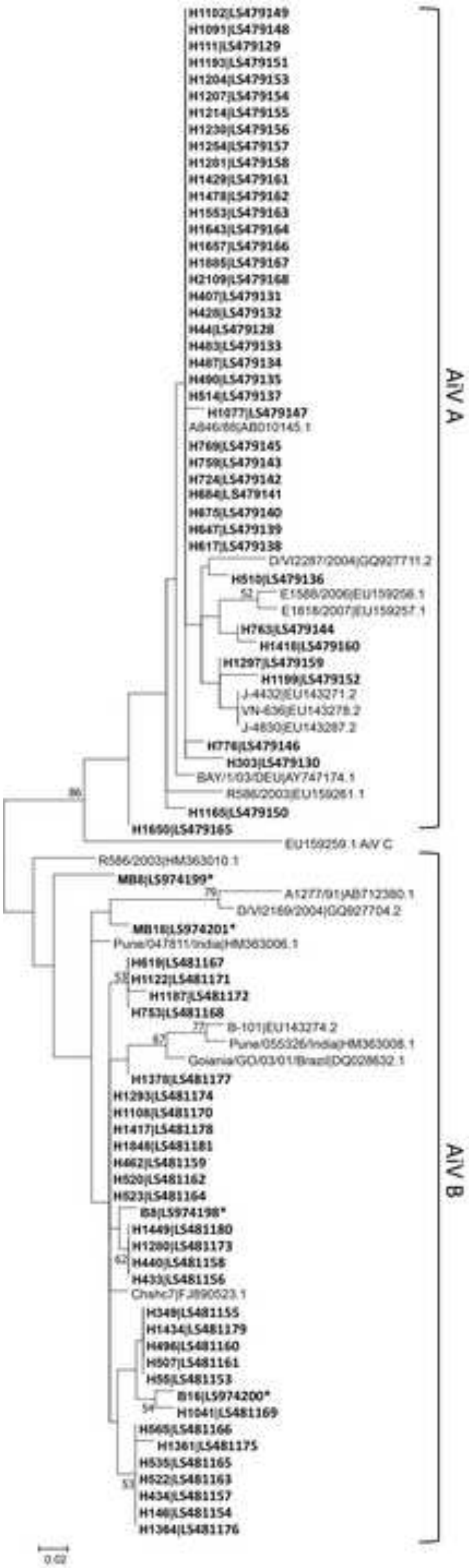

No. of positive samples
40
35
30
25
20
15
10
5
0
July
August
September
October
November
December
January
February
March
April
May
June
2010
2011
Year

Figure 4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H1193|LS479151
H1204|LS479153
H1207|LS479154
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H1230|LS479156
H1254|LS479157
H1281|LS479158
H1429|LS479161
H1478|LS479162
H1553|LS479163
H1643|LS479164
H1657|LS479166
H1885|LS479167
H2109|LS479168
H407|LS479131
H428|LS479132
H44|LS479128
H483|LS479133
H487|LS479134
H490|LS479135
H514|LS479137
H1077|LS479147
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H675|LS479140
H647|LS479139
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52
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H1199|LS479152
J-4432|EU143271.2
VN-636|EU143278.2
J-4830|EU143287.2
H776|LS479146
H303|LS479130
BAY/1/03/DEU|AY747174.1
R586/2003|EU159261.1
H1165|LS479150
H1650|LS479165
86
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AiV A
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79
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D/VI2169/2004|GQ927704.2
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H1122|LS481171
H1187|LS481172
H753|LS481168
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B-101|EU143274.2
67
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Goiania/GO/03/01/Brazil|DQ028632.1
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H1280|LS481173
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H440|LS481158
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AiV B
0.02

EPIDEMIOLOGY OF AICHI VIRUS IN FECAL SAMPLES FROM OUTPATIENTS WITH ACUTE GASTROENTERITIS IN NORTHWESTHERN SPAIN.

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Supplementary Material

Supplementary Table 1. Distribution of AiV mono and mixed infections in the different age groups.

Agent	**Age group**							**Total**
	0-2	**3-5**	**6-12**	**13-18**	**19-59**	**>60**	**Unclassified**	
AiV	17	5	7	3	21	17	2	72
AiV + SaV	2	3	1	0	4	1	0	11
AiV + NoV GI	2	0	0	0	3	1	1	7
AiV + NoV GII	5	2	0	0	0	0	0	7
AiV + NoV GI + SaV	3	0	1	0	0	1	0	5
AiV + NoV GII + SaV	4	0	0	0	1	0	0	5
AiV + *Campylobacter*	3	0	0	0	1	0	0	4
AiV + *Salmonella*	1	0	0	0	2	0	0	3
AiV + *Aeromonas*	1	0	0	0	0	1	0	2
AiV +NoV GI + SaV + *Campylobacter*	1	1	0	0	0	0	0	2
AiV + *Cryptosporidium*	1	0	0	0	0	0	0	1
AiV + SaV + *Campylobacter*	1	0	0	0	0	0	0	1
AiV + SaV + *Yersinia enterocolitica*	1	0	0	0	0	0	0	1
AiV + NoV GII + *Yersinia enterocolitica*	1	0	0	0	0	0	0	1
AiV + NoV GII + SaV + *Campylobacter*	0	0	1	0	0	0	0	1
AiV +NoV GI + SaV + *Bacillus cereus*	0	0	0	0	1	0	0	1
TOTAL	43	11	10	3	33	21	3	124

Author's contributions

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

JLR designed the work. ER and MFV performed the experiments. ER and JLR analyzed the data. ER and JLR wrote the paper. All authors revised the manuscript, read and approved the final draft. JLR supervised the study.