Ten different viral agents infecting and co-infecting children with acute gastroenteritis in Southern Italy: Role of known pathogens and emerging viruses during and after COVID-19 pandemic

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Abstract
Acute gastroenteritis (AGE) represents a world public health relevant problem especially in children. Enteric viruses are the pathogens mainly involved in the episodes of AGE, causing about 70.00% of the cases. Apart from well-known rotavirus (RVA), adenovirus (Adv) and norovirus (NoV), there are various emerging viral pathogens potentially associated with AGE episodes. In this study, the presence of ten different enteric viruses was investigated in 152 fecal samples collected from children hospitalized for gastroenteritis. Real time PCR results showed that 49.3% of them were positive for viral detection with the following prevalence: norovirus GII 19.7%, AdV 15.8%, RVA 10.5%, human parechovirus (HPeV) 5.3%, enterovirus (EV) 3.3%, sapovirus (SaV) 2.6%. Salivirus (SalV), norovirus GI and astrovirus (AstV) 1.3% each, aichivirus (AiV) found in only one patient. In 38.2% of feces only one virus was detected, while co-infections were identified in 11.8% of the cases. Among young patients, 105 were ≤5 years old and 56.0% tested positive for viral detection, while 47 were >5 years old with 40.0% of them infected. Results obtained confirm a complex plethora of viruses potentially implicated in gastroenteritis in children, with some of them previously known for other etiologies but detectable in fecal samples. Subsequent studies should investigate the role of these viruses in causing gastroenteritis and explore the possibility that other symptoms may be ascribed to multiple infections.

KEYWORDS
adenovirus, astrovirus, epidemiology, evolution, genetic variability, human rotavirus, infection, seasonal incidence, virus classification
INTRODUCTION

Infectious acute gastroenteritis (AGE) represents a world public health relevant problem being an important cause of children mortality in developing countries and the main cause of pediatric morbidity worldwide. The pathogens mainly involved in the episodes of AGE are the enteric viruses, which account for about 70% of the cases and are responsible every year for millions of children infections. Gastroenteritis is usually characterized by mild symptoms lasting a couple of days, with symptoms including diarrhea, abdominal pain, nausea, vomiting. Most of the AGE episodes are therefore not notified since not requiring medical support. Only in cases of hospitalization, the cause of the disease is further investigated. The viruses usually investigated are the pathogens more commonly associated with AGE: noroviruses, adenovirus, and rotavirus. Human norovirus (NoV) is the most important cause of nonbacterial AGE worldwide, affecting all age classes. Noroviruses are grouped into genogroups (GI-GX), further sub-classified in genotypes. The genogroups GI and GII cause disease in humans, GII is the predominant and GI is frequently detected in the environment and mussels. Group A rotavirus (RVA), is one of the leading causes of AGE in infants and children worldwide. In the countries with high vaccine coverage, the incidence has decreased but it is still a major cause of AGE in children. Adenovirus, double-stranded DNA viruses, comprises a wide heterogeneous group of different serotypes; serotypes 40 and 41 of species F, are the enteric AdV responsible of AGE in children.

As a matter of fact hospitalized children are often discharged without an infective agent diagnosis and this diagnostic gap let us to speculate that there could be the involvement of other pathogens, as indicated by recent surveillance studies which showed remarkable prevalence of less known viruses among which: astrovirus (AstV), sapovirus (SaV), aichivirus (AiV), human parechovirus (HPeV), salivirus (SalV) and enterovirus (EV). As to AstV and SaV, various studies have already referred to these viruses as pathogens involved in AGE. What's more, data on their environmental circulation in the region Campania, indicated accountable prevalence of the two viruses during the past 8 years, suggesting the possible presence of these pathogens in human feces from our territories.

AdV has been reported as implicated in various cases of gastroenteritis around the world. In some papers, the virus is described as co-infecting agent causing subclinical infections while some other studies ascribe to AstV symptoms of AGE varying from mild to severe. SaV has been identified in feces of children affected by gastroenteritis in previous research carried out in United States and in Australia even though a relationship between virus and AGE episodes has not been clearly established yet. In Italy, its environmental circulation has been evaluated as accountable since found in about 1/3 of the urban sewage samples analysed. These results lead the authors to hypothesize the probable involvement of the virus in cases of gastroenteritis in adults. EV and HPeV infect humans, causing mild respiratory and gastroenteric symptomatology as well as severe disease. HPeV, neglected by the scientific community in the past, is now raising awareness all over the world because of continuous reports of its circulation with different clinical manifestations. The most commonly circulating HPeV is HPeV-1, which principally causes mild gastrointestinal and respiratory symptomatology: the disease can however become more severe in young children. HPeVs are, (together with enterovirus) the second most important cause of viral sepsis-like illness and meningitis in infants, primarily caused by HPeV-3, the most pathogenic HPeV type. EVs are responsible for a wide variety of syndromes, including febrile exanthemas, respiratory infections, aseptic meningitis, encephalitis, paralytic illness, myocarditis, gastroenteritis and others. EVs involvement in AGE is strongly suspected by their detection in infected patients. However, the role of these viruses as pathogens causing AGE is still not fully understood.

In the present study we determined, by Real time PCR, the prevalence of ten different viruses in a pediatric population of Southern Italy hospitalized with AGE. Besides the RVA, AdV and noroviruses, (that we called known pathogens, KP) other six less-known viruses (that we called emerging pathogens, EP) were investigated: AstV, SaV, AiV, HPeV, SalV and EV. The viruses identified underwent characterization by phylogenetic analysis. To trace a picture of the epidemiological situation, data on co-infection, seasonality and symptomatology were also collected and analysed taking in due account the influence of the long-term impact of the COVID-19 pandemic.

MATERIALS AND METHODS

2.1 Ethical approval and study design

Ethical approval was not required for our study since fecal samples were collected for hospital routine analysis from children hospitalized for gastroenteritis. Samples were further investigated within a National Project (IZSME 07/2019 financed by the Ministry of Health). Parents of children provided written informed consent after being briefed on the purpose of the study and of their right to keep information confidential.

2.2 Materials

From March 2021 to March 2023, feces from children hospitalized with gastroenteritis at the Santobono pediatric hospital of Naples were collected and when tested negative for bacterial pathogens and negative to SARS-CoV-2 (by swab rapid test), were sent to IZSM for virological investigations. In details a total of 152 stool samples were analysed (45 in 2021, 102 in 2022 and five in 2023) from pediatric patients suffering with at least one of the following symptoms: fever, abdominal pain, diarrhea, vomiting, bloody diarrhea. Children were 79 males and 73 females with a ratio of 1.08 (M/F) and varied in age from 3 weeks to 13 years old (mean: 4 years old; median age: 2). Samples from 2023, since only...
five, were grouped with those from 2022 when performing viral year prevalence analysis.

### 2.3 Viral nucleic acids extraction procedure

Nucleic acids extraction was carried out using the King Fisher Flex System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the MagMax Viral Pathogen kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer instructions. Before nucleic acid extraction, 100 mg of each stool sample were suspended in 0.9 ml PBS and pretreated as already described. Next, 200 µl of pretreated samples were artificially contaminated with 10 µl of murine norovirus, which was used as an external positive control (EPC) for nucleic acid extraction to monitor the presence of inhibitors in accordance with Amoroso et al. 2021. Results were analyzed as follows: if the threshold cycle (Ct) of the positive control (EPC) in the eluted sample was comparable to that of the EPC in the negative process control (NPC), the sample was analyzed as undiluted. If, instead, the difference between the two Cts was at least 3 or a multiple of 3, all the analyses were carried out on the sample diluted 1:10 or more. The NPC was prepared using 200 µl of PBS instead of sample. Extracted nucleic acids were eluted in 80 µl elution buffer and immediately analyzed by Real-Time PCR/RT-PCR or stored at −80°C until further processing.

### 2.4 Detection of viruses by real time PCR and real time RT PCR

Viral detection was performed as described in the literature (see Table S1). The reactions (a separate real time PCR reaction for each target) were carried out on a QuantStudio 5 Real-Time PCR thermalcycler (Thermo Fisher scientific, Waltham, Massachusetts, USA) with the TaqPath-ID™-1 Master Mix RT-PCR kit (Applied Biosystems by Thermo Fisher Scientific) for RNA virus detection and TaqMan Universal PCR Master Mix (Applied Biosystems by Thermo Fisher Scientific) for DNA virus detection. The reactions were performed in a final volume of 25 µl with 5 µl of nucleic acids sample.

### 2.5 Droplet digital PCR (ddPCR) for viral load calculation

Droplet digital PCR was carried out on Real time positive samples using QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA). The reaction was performed in 22 µl of final volume containing 5 µl of template along with Supermix for Probes no dUTP (Bio-Rad Laboratories) for DNA viruses, or one-step RT-ddPCR Advanced Kit for Probes (Bio-Rad Laboratories) for RNA viruses, 0.9 µM of each primer, 0.25 µM of probe and nuclease-free water to reach the final volume. Primers and probes sequences were the same used for Real-time PCR (see Table S1). Each sample was partitioned into approximately 20,000 nanoliter-sized droplets with AutoDG automated droplet generator (Bio-Rad Laboratories) using QXOx AutoDG oil for Probes. Next, the plate was sealed with pierceable foil heat-sealed (Bio-Rad) at 180°C using PX1 PCR plate sealer (Bio-Rad Laboratories) and PCR amplification was carried out in T100 Thermal Cycler (Bio-Rad Laboratories) with the following thermal profile: reverse transcription (not carried out for DNA viruses) at 50°C for 60', enzyme activation at 95°C for 10', 40 cycles of 94°C for 30' and 60°C for 60' followed by a final elongation step at 98°C for 10'. The plate was then loaded on the QX200 Droplet Reader (Bio-Rad Laboratories). QuantaSoft software was used to calculate target concentration, expressed in copies/µl. Samples were considered positive when at least three droplets containing the target were present, as per manufacturer’s indications. Final concentration results were expressed as genome copies/g stool.

### 2.6 Sequencing and phylogentic analysis of positive samples

Real-time PCR positive samples with a Ct ≤ 30 were further processed for sequencing. The One-Step RT-PCR kit (Qiagen, Hilden, Germany) was used for RNA virus detection, followed by nested or semi-nested PCR using GoTaq Master Mix (Promega, MI, Italy). For AdV, the first PCR and the nested PCR were performed with the GoTaq Master Mix (Promega, MI, Italy). All PCRs and sequencing reactions were carried out according to already established protocols (Table S2). Nucleotide sequencing of amplified genome fragments was performed at Eurofins Genomics (Ebersberg, Germany) and the obtained sequences were uploaded into the NCBI database (https://www.ncbi.nlm.nih.gov) after editing with the ChromasPro2.23 software (Technelysium, Queensland, Australia). Nucleotide and amino acid sequence similarity searches were carried out using the BLAST (Basic Local Alignment Search Tool) server on the NCBI GenBank database: http://www.ncbi.nlm.nih.gov/genbank/index.html. Norovirus genotypes and variants were assigned using the public database NoroNet typing tool (http://www.rivm.nl/mpf/norovirus/typingtool). The maximum likelihood (ML) phylogenetic trees were constructed as suggested by the MEGA6 software model test (http://www.megasoftware.net), based on 1000 bootstrap replications. Sequences were submitted under the following accession numbers: OR077074-OR077091; OR077092; OR058538-OR058545; OR031113-15; OR065074-OR065089; OR087826; OR047820-25.

### 2.7 Statistical analysis

The percentage (with 95% CI) of the results was calculated. Comparison of proportions of categorical variables was carried out by Fisher’s exact test. Means were compared by unpaired t-student test. Statistical analysis was performed using GraphPad Software InStat 3, (Dotmatics, Atlanta, GA, USA). P values < 0.05 were considered statistically significant.
3 | RESULTS

3.1 | Viral prevalence and quantitative viral load

During the 2-year study period, 152 fecal samples were examined (45 in 2021, 102 in 2022, and five in 2023) from pediatric patients hospitalized for AGE. The overall results showed that 75/152 patients (49.3%) were infected by at least one viral pathogen, with 35/60 (58.3%) fecal samples found positive during autumn/winter season (A/W), 40/92 (43.5%) during spring/summer (S/S) season.

Examining results of single virus detected (see Figure 1) the most frequently identified during the 2 years were norovirus GII (30/152 positive samples, 19.7%) and AdV (24/152, 15.8%). RVA showed instead a prevalence of 10.5% (16/152), followed by HPeV (8/152, 5.3%), EV (5/152, 3.3%) and SaV (4/152, 2.6%). SalV, norovirus GI, and AstV were found in two fecal samples each, (prevalence of 1.3%) while only one of the samples analysed tested positive for aichivirus. Results related to the single years showed that, in 2021 norovirus GI, AIv AstV and EV were not identified. RVA was the most prevalent virus (7/45, 15.5%) followed by norovirus GII, AdV and HPeV found with a prevalence of 4.4% each (2/45 positive samples). The most prevalent virus in 2022 was instead norovirus GII (28/107, 26.2%) followed by AdV (22/107, 20.6%) and RVA (9/107, 8.4%) (Figure 1).

### Table 1

<table>
<thead>
<tr>
<th>Detected viruses</th>
<th>Lower concentration (copies/g feces)</th>
<th>Higher concentration (copies/g feces)</th>
<th>Average concentration&lt;sup&gt;a&lt;/sup&gt; (copies/g feces)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdV</td>
<td>3.4 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.6 × 10&lt;sup&gt;14&lt;/sup&gt;</td>
<td>9.4 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>NoV GI&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.6 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.7 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>NoV GII</td>
<td>2.8 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.3 × 10&lt;sup&gt;13&lt;/sup&gt;</td>
<td>8.5 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>RVA</td>
<td>1.5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5.4 × 10&lt;sup&gt;11&lt;/sup&gt;</td>
<td>3.2 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>EV</td>
<td>4.0 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5.1 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>6.0 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>SaV</td>
<td>3.4 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.7 × 10&lt;sup&gt;10&lt;/sup&gt;</td>
<td>6.6 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>AIv&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>1.6 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>SaVV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.7 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.7 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>HPeV</td>
<td>4.0 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.0 × 10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>5.2 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>AstV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.3 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>2.9 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: AdV, adenovirus; AIv, aichivirus; AstV, astrovirus; EV, enterovirus; HPeV, human parechovirus; NoV GI, norovirus GI; NoV GII, norovirus GII; RVA, rotavirus; SaV, sapovirus; SaIV, salivirus.

<sup>a</sup>Two samples quantified.
<sup>b</sup>One sample quantified.
<sup>c</sup>Geometric mean.
Samples positive to Real Time PCR underwent ddPCR to determine the viral load of each virus detected. As reported in Table 1 the virus exhibiting the highest average concentration (viral genome copies/g feces, gc/g) was AdV ($9.4 \times 10^9$ gc/g), followed by norovirus GII ($8.5 \times 10^9$ gc/g), RVA ($3.2 \times 10^9$ gc/g), SaIV ($2.65 \times 10^8$). The remaining viruses showed instead an average viral load below $10^8$ gc/g.

### 3.2 Virus detection in the different age categories during and after COVID-19 pandemic

Results of the viral infections, due to at least one virus detected, were further analysed with respect to children age, according to which the young patients were divided into three categories: 0–2 (including also the children >2 but <3), 3–5 and >5 years old. Furthermore data were analysed considering the effects of restriction measures due to COVID-19, which were still on during 2021 and were almost all eliminated during 2022 (Table 2). Very interestingly, a significant (p < 0.01) difference was observed when comparing total positive children found in 2021 and in 2022. In details about one/third of samples resulted positive in 2021 (14/45, 31.1%) while in 2022 more than half of children feces tested positive to at least one viral pathogen (57.0%, 61/107). Additionally, in all the three age groups viral positivity increased from 2021 to 2022 with statistically significant prevalence increment in primary school age (>5) group (p < 0.05).

Further results were obtained analyzing single viral prevalence in the three age categories. In AdV group, a significant (p < 0.05) difference was observed when positive children of 0–2 age category were compared with those >5 years. Indeed, AdV was the virus most frequently identified in the 0–2 category patients (21.3%), while its involvement in the gastroenteritis events decreased with age reaching 6.4% in children >5 years old. Norovirus GII was the most prevalent pathogen in the other two groups 3–5 (28.0%) and >5 years (17.0%). RVA ranked as third virus most frequently identified in the three age categories, with prevalence of 7.5% in the youngest (0–2 years), and respectively 16.0% and 12.7% in the remaining two categories (3–5 and >5 years old). Within the emerging viruses, HPeV was the most prevalent in the younger children (0–2 years, 6.3%) and in the elder (>5 years, 4.3%) while in the group 3–5 no difference among the emerging viruses prevalence was observed. Table 3

#### 3.3 Analyses of single infections and co-infections

Results showed that in 58/75 (77.3%) positive stool samples a single virus (single infection) was detected, while 17/75 (22.7%) presented coinfection, being positive for two or more viruses. The co-infections were mainly observed in the 0–2 age group (12/17, 70.6%, Figure 2A) and norovirus GII - HPeV (four children) and norovirus GII – AdV (four children) were the viruses most frequently associated. Co-infections were all registered in 2022, with only one co-infection observed in 2021 (a 1-year-old child suffering with diarrhea and dehydration resulted positive to both norovirus GII and HPeV). In 2022, 16/61 (26.2%) patients were infected by more than one virus, usually two, making exception a 4-months old female, affected by vomiting, found simultaneously positive to four enteric viruses: NoV GII, RVA, SaV, and EV.

As to single infections and pathogens involved, results showed that they were mainly caused by KP (noroviruses, AdV and RVA) in all the three age groups investigated with percentages ranging from 77.8% to 87.5% (Figure 2B). All viruses belonging to the emerging group (AiV, AstV, EV, HPeV, SaV, SaV), with the exception of AiV, were identified as causing single infections mainly in the age 0–2 years, while single infections in children >3 years old were addressed only to EV, SaV and HPeV (Figure 2C).

#### 3.4 Infections with KP (AdV, NoV GI, NoV GII, RVA)

AdV was identified in feces of children from 3 months to 7 years old who were mostly (18/24, 72.0%) affected by diarrhea in some cases profuse and persistent. When looking at seasonality, AdV positive cases were 11 during A/W season (n = 62) and 13 during S/S (n = 92), see Table 4, with a not statistical difference in the prevalence observed. Among patients, 16/24 showed AdV as single infection, while the other eight cases were characterized by the copresence of

### Table 2 Total viral positivity (to one or more pathogens) referred to children divided for age and year of hospitalization.

<table>
<thead>
<tr>
<th>Age category (years)</th>
<th>Positivity to at least one virus</th>
<th>2021</th>
<th>2022</th>
<th>Total</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–2 (n = 80)</td>
<td></td>
<td>8/21 (38.1%)</td>
<td>31/59 (52.5%)</td>
<td>39/80 (48.8%)</td>
<td>CI 38.1–59.5</td>
</tr>
<tr>
<td>3–5 (n = 25)</td>
<td></td>
<td>2/5 (40.0%)</td>
<td>15/20 (75.0%)</td>
<td>17/25 (68.0%)</td>
<td>CI 48.3–82.9</td>
</tr>
<tr>
<td>&gt; 5 (n = 47)</td>
<td></td>
<td>4/19 (21.1%)</td>
<td>15/28 (53.6%)</td>
<td>19/47 (40.4%)</td>
<td>CI 27.6–54.7</td>
</tr>
<tr>
<td>Total (n = 152)</td>
<td></td>
<td>14/45 (31.1%)</td>
<td>61/107 (57.0%)</td>
<td>75/152 (49.3%)</td>
<td>CI 41.5–57.2</td>
</tr>
</tbody>
</table>

Abbreviation: CI, confidence interval (95%).

*aIncludes also samples (five) collected in the first months of 2023.*
### TABLE 3
Prevalence of known and emerging viruses in feces of children divided for age group.

<table>
<thead>
<tr>
<th>Age category (years)</th>
<th>Viruses total positivity</th>
<th>KP positivity</th>
<th>EP positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total/age</td>
<td>ADV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NOV GI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.6–31.5 CI</td>
<td>0.0–7.4</td>
</tr>
<tr>
<td>3-5 (&lt;i&gt;n = 25&lt;/i&gt;)</td>
<td>17</td>
<td>4 (16.0%) CI</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.8–35.3 CI</td>
<td>0.0–12.1</td>
</tr>
<tr>
<td>&gt;5 (&lt;i&gt;n = 47&lt;/i&gt;)</td>
<td>19</td>
<td>3 (6.4%) CI</td>
<td>1 (21.2%) CI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6–17.8 CI</td>
<td>8.6–30.4</td>
</tr>
</tbody>
</table>

Abbreviations: ADV, adenovirus; AIV, aichivirus; AstV, astrovirus; CI, confidence interval (95%); EP, emerging pathogens; EV, enterovirus; HPeV, human parechovirus; KP, known pathogens; n.d., not detected; NoV GI, norovirus GI; NoV GII, norovirus GII; RVA, rotavirus; SaV, sapovirus; SalV, salivirus.

<sup>a</sup>p < 0.05 (*) in the comparison between 0 and 2 versus >5 categories from ADV positive samples.

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**3.5 Infections with EP (AstV, EV, HPeV, SaV, SalV)**

AstV was only identified in two fecal samples collected during spring 2022. Two males of 11 weeks and 11 months complaining with strong abdominal pain and no other possible case was hypothesized by medical staff during the infections event. In the two cases, its viral load was 1.3 x 10<sup>9</sup> and 6.0 x 10<sup>8</sup> gc/g.

EV was found in fecal samples in three patients (1, 4, and 9 years old). EV was not detected in children 2, 3, and 5 years old.

HPeV was identified in eight cases (two in 2021 and six in 2022). HPeV was identified in eight cases (two in 2021 and six in 2022).

**Results on RVA showed that the virus infected patients with age ranging from 4 months to 12 years old (mainly 13/16 patients) causing abdominal pain and fever accompanied by diarrhea.** In both episodes, no other pathogen was uncovered.

**Children infected with norovirus GII ranged from 1 month to 12 years old; almost all affected by diarrhea accompanied by vomiting.** There was an extremely significant difference (6 < 0.001) in 18 of the positive samples collected in A/W (40.0%) and in S/S (6.5%).

**RVA was the sole infecting agent in the 14 out of 16 cases described.** Three children suffered only with vomiting. RVA cases were only observed in HPeV (four samples), ADV (four samples), and AstV (two samples). However, the observed difference was higher (gc/g) in single infections (1.1 x 10<sup>9</sup> gc/g) with respect to co-infections (1.7 x 10<sup>9</sup> gc/g; Table 1): interestingly, the average viral load was higher (gc/g) in single infections (1.1 x 10<sup>9</sup> gc/g) with respect to co-infections (1.7 x 10<sup>9</sup> gc/g; Table 1). Results on RVA showed that the virus infected patients with age ranging from 4 months to 12 years old (mainly 13/16 patients) causing abdominal pain and fever accompanied by diarrhea. In both episodes, no other pathogen was uncovered. The virus was identified in patients with vomiting and abdominal pain./n

**Infections with another virus among norovirus GII, HPeV, SaV, RVA and SalV.** HPeV showed a very high yield, (8.3 x 10<sup>12</sup> and 1.6 x 10<sup>14</sup> gc/g) in two fecal samples belonging to two females (2 and 5 years old), both suffering with profuse diarrhea and high fever.

Norovirus GII was revealed only in two fecal samples (2 and 8 years old) in 2022, during the cold season and always in co-infections with another virus (HPeV or norovirus GII). However, infection with another virus (HPeV or norovirus GII) was observed in 12 patients suffering with symptoms like vomiting, abdominal pain, fever. As shown in Table 4, there was an extremely significant difference (6 < 0.001) between the positive samples collected in A/W (40.0%) and in S/S (6.5%).

**Norovirus GII exhibited in both the cases higher gc/g concentration with respect to the co-infecting pathogen: 1.6 x 10<sup>9</sup> gc/g versus 1.4 x 10<sup>7</sup> gc/g and 2.6 x 10<sup>6</sup> gc/g versus 1.62 x 10<sup>5</sup> gc/g, in two children infected with another virus among norovirus GII, HPeV, SaV, RVA and SalV.**
FIGURE 2  Infection rates in the three age categories identified. (A) Prevalence of single infections (red) and mixed infections (blue). (B) Prevalence of single infections caused by known pathogens (KP) and emerging pathogens (EP). (C) Within the single infection positive samples number of cases for each virus (known or emerging) within the age category indicated.
TABLE 4 Viruses detected in children feces (n = 152) collected in autumn/winter (A/W) n = 60 or in spring/summer (S/S) n = 92.

<table>
<thead>
<tr>
<th>Viruses detected</th>
<th>Positive samples</th>
<th>Positive samples in A/W (60)</th>
<th>Positive samples in S/S (92)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdV</td>
<td>24 (15.8%) CI 10.8-22.5</td>
<td>11 (18.3%) CI 10.4-30.1</td>
<td>13 (14.1%) CI 8.3-22.8</td>
</tr>
<tr>
<td>NoV GI</td>
<td>2 (1.3%) CI 0.1-5.0</td>
<td>2 (3.3%) CI 0.3-12.0</td>
<td>-</td>
</tr>
<tr>
<td>NoV GII</td>
<td>30 (19.7%) CI 14.2-26.8</td>
<td>24 (40.0%) CI 28.6-52.7</td>
<td>6 (6.5%) CI 2.8-13.8</td>
</tr>
<tr>
<td>RVA</td>
<td>16 (10.5%) CI 6.5-16.5</td>
<td>n.d.</td>
<td>16 (17.4%) CI 10.9-26.5</td>
</tr>
<tr>
<td>AiV</td>
<td>1 (0.7%) CI 0.0-4.0</td>
<td>n.d.</td>
<td>1 (1.1%) CI 0.0-6.5</td>
</tr>
<tr>
<td>AstV</td>
<td>2 (1.3%) CI 0.1-5.0</td>
<td>n.d.</td>
<td>2 (2.2%) CI 0.1-8.1</td>
</tr>
<tr>
<td>EV</td>
<td>5 (3.3%) CI 1.2-7.7</td>
<td>1 (1.7%) CI 0.0-9.7</td>
<td>4 (4.4%) CI 1.4-11.0</td>
</tr>
<tr>
<td>HPEV</td>
<td>8 (5.3%) CI 2.5-10.2</td>
<td>5 (8.3%) CI 3.2-18.5</td>
<td>3 (3.3%) CI 0.7-9.6</td>
</tr>
<tr>
<td>SaV</td>
<td>4 (2.6%) CI 0.8-6.8</td>
<td>1 (1.7%) CI 0.0-9.7</td>
<td>3 (3.2%) CI 0.7-9.6</td>
</tr>
<tr>
<td>SalV</td>
<td>2 (1.3%) CI 0.1-5.0</td>
<td>1 (1.7%) CI 0.0-9.7</td>
<td>1 (1.1%) CI 0.0-6.5</td>
</tr>
</tbody>
</table>

Abbreviations: AdV, adenovirus; AiV, aichivirus; AstV, astrovirus; CI, confidence interval (95%); EV, enterovirus; HPEV, human parechovirus; n.d., not detected; NoVGI, norovirus GI; NoVGII, norovirus GII; RVA, rotavirus; SaV, sapovirus; SalV, salivirus.

*p-value < 0.001 (***') in the comparison between A/W versus S/S groups from NoVGII and RVA positive samples.

found associated with norovirus GII in five cases, with AdV or norovirus GI in one case; while it was identified as unique pathogen in two children of 13 and 1 years old suffering with strong abdominal pain. In particular in the feces of the latter child, the virus exhibited a notable concentration value, (4.0 × 10¹² gc/g) and, since no other enteric pathogen was identified, the AGE episode was addressed to HPEV.

SaV was found in four fecal samples (one in 2021 and three in 2022), in two as the sole infecting agent: one from 2021 (a 1-year-old male with appendicopathecy) and one from 2022 (3 years old female with abdominal pain) with a maximum viral load of 4.5 × 10¹⁰ gc/g (see Table 1).

SaV was identified only in two fecal samples, belonging to 10-months and 7-years old children and collected respectively in 2021 and 2022. In both the patients, suffering with diarrhea, the virus was found at a similar concentration (2.7 × 10⁹ gc/g), as a single virus in the ten months old baby, and as co-infection with AdV (2.9 × 10⁷ gc/g) in the other patient.

3.6 | Viral characterization and phylogenetic analysis

Not all the identified viruses have been successfully typed probably due to high Ct-values representing low viral loads. In 2021 seven HuAdF serotype 41 (PO8, PO9, PO10 PO12, PO24, PO25, PO28) and three HuAdVB serotype 3 (PO4, PO30, PO31) were identified. In 2022, six HuAdVF serotype 41 (PO177, PO179 PO180, PO188, PO191, PO198) and three HuAdVC of serotype 2 (PO138-139) and serotype 1 (PO172), were characterized (Figure 3A, B). Sequenced strains of AdV41 formed two clusters, one of which (including strains PO9, PO28, PO25) showed 100% n.i. to each other and also identical to strains identified in Italy in 2009 in patients, water, and waste34-36 (Figure 3A).

Norovirus sequencing of capsid fragments (300 bp) confirmed the detection of the sole GI genogroups belonging to different genotypes. Based on the capsid fragment (ORF2) sequence classification, the GII.3 (n = 10, two in 201 and eight in 2022) was the most frequent, followed by GII.2 (n = 3) (Figure 4A). The GII.3 strains were closely related to each other 99%–100% nt.id. For seven samples the ORF1 (RdRp fragment) was also sequenced to monitor events of recombination (Figure 4B). For two samples (PO129, PO130), the recombination event of the GII.3 (ORF2) with GILP12 (RdRp sequence) was confirmed by sequencing of the junction ORF1-ORF2. The fecal sample PO133 (Figure 4A) classified as GII.3 based on ORF2 fragment, revealed the GILP30 in the RdRp sequence fragment (Figure 4B) but could not be confirmed as recombinant since the whole junction could not be sequenced. More sporadically, once in the whole survey other genotypes were also detected such as GII.13, GII.1 in combination with GILP33 and two cases positive for GII.4.

Sequenced human RVA strains were represented by two G genotypes (G1, G3) and one P genotype P[8]. The G-P genotypes detected comprising of G1P[8] (PO121) and G3P[8] (PO187, PO188) are two common combinations. The G3 sequences showed that they are all equine-like G3P[8] strains.

One AstV positive fecal sample (PO140) was sequenced and characterized. The detected AstV strain belonged to the Mamas-trovirus 1 genotype (MAstV 1), serotype 1 (HAstV 1), showing the highest nucleotide identities (100% n.t. id.) with AstV strains detected in the same area in both patients (MK041036, MK041037) and sewage waste waters (MW842776, MW842777), in 2017 and 2019, respectively.15,27
Three fecal samples (PO130, PO133, and PO188) tested positive for parechovirus have been successfully characterized. Nucleotide sequencing revealed the presence of human PeV strains belonging to the PeV-A1 genotype, displaying nt. id. ranging between 90.2% and 92.6% each other. Parechovirus strains were previously detected in Italy in children but were not so related with strains detected in this study, displaying 95% nt.id (MF919559.1)\(^3\) (Figure 5).

Probably due to low amount of RNA recovered from feces (average \(1.1 \times 10^3\)gc/g), only one SaVs positive was sequenced and classified as Gl.1.

4 | DISCUSSION

The aim of the present study was to characterize enteric viral infections in pediatric patients hospitalized for acute gastroenteritis, also evaluating difference due to COVID-19 pandemic both during and post-restriction measures. The research was carried out on hospitalized children from 3 weeks to 13 years old; the majority of patients being ≤5 years old (69.1%) probably because parents of younger kids are more worried from symptomatology and more easily bring their children to hospital due to an age correlated severity of gastrointestinal symptoms.\(^3\) The results obtained enable to evaluate the circulation of viruses causing gastroenteritis in children. Comparing our results with previous studies, viral prevalence in the 2 years (49.3%) was consistent with that calculated in a 11-year surveillance carried out on four (KP) viruses in Sicily (49.8%) on children <5 years old\(^4\)\(^-\)\(^8\); it is instead much higher than that reported in a similar study\(^1\) carried out in Northern Italy (29.6%). However, analyzing the 2 years separately, in 2022 a significantly higher prevalence (\(p < 0.01\)) was registered with respect to 2021 (57.0% vs. 31.1%). This difference may be due to COVID-19 social restrictions imposed by sanitary authority in 2021 with the aim to limit SARS-CoV-2 circulation. The higher attention to restrictions in 2021 also very likely influenced the circulation of the viruses transmissible from person to person, such as enteric viruses. Confirming this, overall
complete elimination of pandemic social distancing and individual protection measures (masks) in 2022 allowed the oro-fecal transmission viruses to re-start their circulation and transmission among humans, as indicated by our data showing very intense viral prevalence in 2022 (57.0%). Similar conclusions were also reached by other authors, who observed that the restriction measures adopted during COVID-19 pandemic to avoid SARS-CoV-2 circulation, also diminished the spread, among children, of other respiratory pathogens. Differently from us these authors compared detection rates of respiratory viruses before and during pandemic while we observed prevalence of gastroenteric viruses during and after pandemic.

Very interestingly, differences in viral positivity increment observed among the three age groups from 2021 to 2022, (Table 2) may reflect the different social interaction of these three age groups during and after COVID-19 pandemic. Indeed, infants belonging to age group 0-2 usually do not go to school or to gymnasium and their source of infection is mainly related to family contacts. Differently, school-age children infections were directly affected by social restrictions which forced them at home. This influence was particularly relevant (and statistically significant) for the children belonging to age group >5 who probably, being elder, have a more intense “social life,” more frequently attending (beyond school) meeting places like cinemas, gymnasium, parties. Social distancing could also explain the different rate of co-infections registered in the 2 years: they were almost completely absent (only one case) in 2021, while accounted for 22.7% of the cases in 2022. As a matter of fact in the last years, viral co-infections have been increasingly described

FIGURE 4   Phylogenetic trees of the norovirus (NoV). The norovirus strains sequenced in this study are highlighted with a filled circle. The maximum likelihood (ML) phylogenetic tree was constructed with the Tamura-3 with gamma distributed and uniform rates parameter model (T92 + G), with 1000 bootstrap repetitions. Bootstrap values under 70% are not shown. (A) Capsid protein gene partial ORF2 nucleotide sequences. (B) RdRp protein gene partial ORF1 nucleotide sequences.
thank to the ability to analyze samples with more sensitive
approaches. Our data, (with 22.7% of coinfected samples), are
within the 5%–50% range reported in the literature. Some
authors argue the possible increment of disease severity as
a consequence of co-infections, especially those related to norovirus. Our results can’t support this hypothesis since we didn’t observe any
difference in symptomatology in patients with mixed infections with
respect to single infections (data not shown).

When examining the individual prevalence of the viruses investigated, we observed 19.7% of the patients, belonging to all
the three age categories investigated, were infected by norovirus GII,
confirming its role as the leading cause of gastroenteritis especially
during the cold season. Results of our study showed that GII.3
was the most frequent genotype causing AGE in infants and young
children. As observed for many norovirus genotypes, the GII.3
undergoes to constant changes mainly linked to frequent event of
recombination, in this study the GII.3 was a recombinant associated
to GII.P12. All detected strains were strictly related to each other
suggesting their frequent circulation in children hospitalized in the
investigated area during 2021–2022. The other genotypes (GII.2;
GII.13) were only detected in a few patients as previously described
in Italy. A deeper sequencing would have been required for further

FIGURE 5 Phylogenetic tree of the parechovirus (HPeV) polyprotein gene partial ORF
nucleotide sequences. The PeV strains sequenced in this study are highlighted with a filled circle.
The maximum likelihood (ML) phylogenetic tree was constructed with the Tamura-3 with gamma
distributed and uniform rates parameter model (T92 + G), with 1000 bootstrap repetitions. Bootstrap values under 70% are not shown.
discussion on circulating strains; however, based on our preliminary results we can confirm their high heterogeneity and their role in norovirus associated AGE in children.

After norovirus GI, AdV was the virus predominantly identified with a prevalence of 15.8%. HuAdVs are a group of pathogens associated with various clinical syndromes (e.g., conjunctivitis, gastroenteritis, and respiratory diseases). The AdVs can be considered typical enteric (such as AdV 41) and non-enteric (such as those mainly causing respiratory symptoms). AdV41, as previously observed in other studies,51–53 is the prevalent circulating serotype in the community under study. Interestingly, we also described the presence of other non enteric AdVs belonging to AdVB3, AdVC1 and AdVC2, as already reported in Italy54 and in other countries.55

Adenovirus species AdV-B of zoonotic origin (types B3, B7, B11, B14, B16, and B21)56 and AdV-C (types C1, C2, C5, and C6) are commonly associated with acute respiratory illnesses.57 As a matter of fact none of the patients tested positive for these viruses showed either respiratory symptoms or other symptoms besides vomiting and diarrhea. Strains belonging to serotype 1 and 2 were previously described in Italian patients, but comparison of sequences did not show a correlation.54 Conversely sequence analyses showed that very similar strains both belonging to AdV41 and AdVB3 were identified in Italy in environmental samples several years ago.34–36

As to the other important enteric virus RVA, prevalence highlighted in this study was 10.5%, (exclusively revealed in the hot season), representing overall a low prevalence with respect to those detected worldwide,5 even if the assessment of a mean RVA prevalence results difficult. The detected low prevalence could have been affected by the months of sampling (92/152 samples were collected in spring-summer months) and by the 59.7% vaccination coverage in the region of sampling (https://www.salute.gov.it/imgs/C_17_tavole_20_10_0_file.pdf). Another interesting point is the ability of RVA to reassert its genomic segments, possibly generating novel variants able to escape the vaccine protection. The spread of the equine-like G3P[8] strains represents an example of strains with human and animal gene segments, and is considered, to date, a genotype largely circulating in different countries with different detection rates.58–60

Even though emerging viruses were less frequently identified with respect to known pathogens and were mainly found associated with norovirus GI, AdV and RVA, in some patients they were the only pathogen identified, suggesting a possible role as causative agent of AGE. SaV and AstV were identified respectively in four and two children as single pathogens in two AGE episodes each. Both the viruses have been already detected with accountable prevalence in the same geographical area of Naples in mussels and wastewater.15,20 Furthermore, sequencing of the AstV strain (HuAstV-1) confirmed the presence of the same viral variant in the same geographical area after 5 years.15 With respect to AstV we did not check the samples for either VA or MLB, since, based on previous studies conducted in Italy, the circulation of VA-1 and MLB-1 is limited.61,62 However, since high seroprevalence (up to 75%–100%) for MLB and VA astroviruses was detected in different countries63,64 and different variants within MLB and VA have been found, future studies will be carried out to assess more properly the epidemiology and role of these viruses in humans in our territories. As to SaV only one strain could be sequenced belonging to GI.1, a genotype frequently detected worldwide and in Italy.65 Interestingly the same strain was identified in wastewaters collected in the same geographical area of Naples10 years ago, confirming again the persistent circulation over time of SaV in Southern Italy.

HPeV was, among the emerging viruses investigated, the most frequently identified (eight cases). It comprises a wide group of viruses, mainly infecting subjects in the first years of life and causing from asymptomatic to gastrointestinal symptoms, or severe symptoms, including sepsis-like syndrome and meningitis.67–70 HPeV infections have been reported in Italy since 2008, mainly investigated as causes of respiratory and neurological symptoms, and mostly due to PeV-A3.70 In this study, HPeV strains belonging to genotype A1 were sequenced, and since patients positive for Parechovirus did not show any other symptom apart from gastroenteric ones, results confirmed the circulation of this genotype also associated to gastrointestinal symptoms.78

Enteroviruses comprise 15 species, with some causing severe diseases in humans. Most infections are mild, but specific serotypes may lead to serious outcomes.7 In this study EV was searched with a protocol able to detect a broad range of enteroviruses, since directed to the highly conserved sequence in the 5’UTR of the genome.71 It was detected in five cases of AGE, in three of them without any other virus. In 2023, an outbreak of enterovirus E11 in European countries resulted in severe cases, including sepsis and fatalities, especially among neonates.72 The EV strain identified in this study in the same period, was further characterized (by sequencing, data not shown) as a coxsackie B5 not the E11 emerging strain.

In this study, interesting results were also obtained from ddPCR evaluation of viral load. The top three viruses with the highest viral loads were AdV, norovirus GI followed by RVA. In other studies, using Ct values, RVA was on the top followed by norovirus and AstV.73 We did not observe any correlation among titres of single infection and co-infection. Establishing biological relevance to viral titre, particularly in the context of co-infection, remains an open question which could be further investigated with a larger number of co-infection cases. However, the viral load is also influenced by timing of specimen collection, which should be taken in account for comparison studies. In our study fecal collection was carried out at the time of hospitalization, which however occurred at different time after the onset of the disease depending on the gravity of the symptomatology and on the age of the patient (younger patients are brought to hospital earlier with respect to older ones).

5 | CONCLUSIONS

Our study was carried out during (2021) and immediately after (2022) the COVID-19 pandemic and the results obtained have been somehow conditioned by the world peculiar situation. This can be
considered an added value since we could evaluate the effect of restriction measures adopted to break SARS-CoV-2 diffusion also on other (enteric and not respiratory) viruses. A preselected bias could be due to the involvement of a single hospital and to the fact that all the patients were hospitalized. The results presented are however very interesting and fascinating and encourage us to carry out further investigations to keep on monitoring viral circulation the “day after” pandemic.

The results obtained have overall showed an intense circulation of enteric viruses and their high degree of involvement in AGE episodes. Even if being often caused by viruses, most gastroenteritis is immediately and often uselessly treated with antibiotics. Using too many antibiotics increases the risk of adverse reactions and results in higher healthcare costs and increased antibacterial resistance.74 On this regard we support the introduction, in the hospitals routine diagnostic activity, of viral protocols for early screening of gastroenteric patients. This would help to avoid the misuse of antibiotics, in accordance with European programs aiming at drug resistance reduction.75

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST STATEMENT
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the supplementary material of this article. Data available in article supplementary material.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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