Published in final edited form as:

Pediatr Infect Dis J. 2016 September ; 35(9): e262–e270. doi:10.1097/INF.0000000000001208.

Narrowing of the Diagnostic Gap of Acute Gastroenteritis in Children 0-6 Years of Age Using a Combination of Classical and Molecular Techniques, Delivers Challenges in Syndromic Approach Diagnostics

Andrej Steyer, PhD^{1,*}, Monika Jevšnik, PhD¹, Miroslav Petrovec, MD, PhD¹, Marko Pokorn, MD, MSc², Štefan Grosek, MD, PhD³, Adela Fratnik Steyer, PhD^{1,**}, Barbara Šoba, PhD¹, Tina Urši , PhD¹, Tjaša Cerar Kišek, PhD¹, Marko Kolenc, BSc¹, Marija Trkov, PhD⁴, Petra Šparl, PhD⁵, Raja Duraisamy, PhD⁶, Ian W. Lipkin, MD, PhD⁶, Sara Terzi , MD², Mojca Kolnik, MD², Tatjana Mrvi , MD², Amit Kapoor, PhD⁷, and Franc Strle, MD, PhD²

¹Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia

²Department of Infectious Diseases, University Medical Centre Ljubljana, Japljeva 2, 1525 Ljubljana, Slovenia

³Department of Pediatric Surgery and Intensive Care, University Medical Centre Ljubljana, Bohoričeva 20, 1000 Ljubljana, Slovenia

⁴Department for Public Health Microbiology, Centre for Medical Microbiology, National Laboratory of Health, Environment and Food, Grablovičeva 44, SI-1000 Ljubljana, Slovenia

⁵Faculty of Organizational Sciences, University of Maribor, Kidričeva cesta 55a, 4000 Kranj, Slovenia

⁶Center for Infection and Immunity, Mailman School of Public Health, Columbia University, New York, NY, USA

⁷The Research Institute at Nationwide Children's Hospital, The Ohio State University, Columbus, OH, USA

Abstract

Background—25%-50% of acute gastroenteritis (AGE) cases remain etiologically undiagnosed. Our main aim was to determine the most appropriate list of enteric pathogens to be included in the daily diagnostics scheme of AGE, ensuring the lowest possible diagnostic gap.

Methods—297 children 6 years of age, admitted to hospital in Slovenia, October 2011 – October 2012, with AGE, and 88 6 year old healthy children, were included in the study. A broad

*Address correspondence to: Andrej Steyer, PhD, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia; Tel: +386 1 543 7459; andrej.steyer@mf.uni-lj.si.

**Current Address: Blood Transfusion Centre of Slovenia, Šlajmerjeva 6, 1000 Ljubljana, Slovenia

We have no conflict of interest to report.

spectrum of enteric pathogens was targeted with molecular methods, including 8 viruses, 6 bacteria and 2 parasites.

Results—At least one enteric pathogen was detected in 91.2% of cases with AGE and 27.3% of controls. Viruses were the most prevalent (82.5% and 15.9%), followed by bacteria (27.3% and 10.2%) and parasites (3.0% and 1.1%) in cases and controls, respectively. A high proportion (41.8%) of mixed infections was observed in the cases. For cases with undetermined etiology (8.8%), stool samples were analyzed with next generation sequencing and a potential viral pathogen was detected in 17 additional samples (5.8%).

Conclusions—Our study suggests that tests for rotaviruses, noroviruses genogroup II, adenoviruses 40/41, astroviruses, *Campylobacter* spp. and *Salmonella* sp. should be included in the initial diagnostic algorithm, which revealed the etiology in 83.5% of children tested. The use of molecular methods in diagnostics of gastroenteritis is preferable because of their high sensitivity, specificity, fast performance and the possibility of establishing the concentration of the target. The latter may be valuable for assessing the clinical significance of the detected enteric, particularly viral pathogens.

Keywords

gastroenteritis; children; syndromic approach; molecular methods; diagnostic gap; enteric pathogens; case-control study; next generation sequencing

Introduction

Acute gastroenteritis (AGE) remains an important cause of mortality among children worldwide. In 2008 AGE in children ranged in third place as a cause of mortality after neonatal disease and pneumonia (1). Many different pathogens, including viruses, bacteria and parasites, are associated with AGE. Their prevalence varies among world regions. Enteric viruses are among the most important pathogens contributing to the high disease burden in developed and developing regions (2). A systematic review of AGE mortality in children revealed that the four major pathogens, rotaviruses, caliciviruses, enteropathogenic and enterotoxigenic *Escherichia coli*, account for more than half of all AGE deaths in children <5 years of age in the world (3). In developed countries, mortality for AGE is low but the incidence of infectious diarrhea remains high, despite better hygiene conditions. In these countries, viruses are more prevalent than bacterial pathogens (3, 4). However, in the last few years, rotavirus vaccine has contributed to a significant reduction of the disease burden, especially in countries with high vaccination coverage (5-8). In such an environment a substantial decrease in rotavirus disease and overall AGE cases was noted, increasing consequently the (relative) importance of other enteric pathogens in AGE (3, 9, 10).

Previous studies have revealed a variable proportion of etiologically undiagnosed AGE cases (diagnostic gap). This might suggest that pathogens other than those already diagnosed could contribute to the overall disease burden (11-13). The rate of etiologically undiagnosed AGE cases varies among published reports, from 25% to 49% (14) or even higher in the non-pediatric population. Newly developed methods enable a syndromic approach in

diagnostics of AGE that efficiently in decrease etiologically undiagnosed cases but, at the same time, increase the proportion of detected mixed infection (15-17).

The main aim of this case-control study was to profile the most important pathogens associated with severe cases of AGE in children, to determine and narrow the diagnostic gap, and to improve the diagnostic approach with the ambition of optimizing the management of such cases. We used broad range molecular testing for this and, subsequently, new molecular techniques, next generation sequencing (NGS), to screen undiagnosed cases of AGE for unspecified, less frequent viral targets. The clinical importance of pathogens was also evaluated.

Materials and methods

Study design

A case-control study was conducted at the University Medical Centre Ljubljana, Department of Infectious Diseases and Department of Paediatric Surgery and Intensive Care, on the etiology of AGE, using broad spectrum detection methods for viruses, bacteria and parasites. The University Medical Centre is located in Ljubljana, the capital of Slovenia, covering the Central Slovenian region, with approximately 536,000 inhabitants, 37,000 (6.9 %) of whom are children up to 6 years of age (<http://www.stat.si/StatWeb/en/home>). This is the area with the highest population density, including the capital, an urban population in smaller settlements and a rural part, also with intensive agriculture.

The enrollment phase of the study started on October 5th, 2011 and was completed on October 11th, 2012. Two groups of children up to six years of age, for whom parental consent was obtained, were included in the study: patients with AGE (AGE group) and a control group. Children with AGE (defined by passing a loose stool at least three times within the last 24 hours, with or without vomiting, lasting for 10 days) were hospitalized at the Department of Infectious Diseases. The control group comprised children who had been hospitalized at the Department of Paediatric Surgery and Intensive Care for an elective surgical procedure, and who had been without clinical signs of acute gastroenteritis for at least 10 days prior to the procedure and 7 days after discharge from the hospital. Information about signs of AGE was obtained by a questionnaire for operated children and their family members. A stool sample was collected from AGE cases and controls (one sample per enrolled subject) immediately after admission and sent to the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana for microbiological examination. Approval for the study was obtained from the Slovenian National Medical Ethics Committee (Nr. 138/07/11).

The severity of illness of children with AGE was assessed using the Vesikari score (18). The parameters used in the scoring system include the duration (in days) of diarrhea and maximum number of diarrhea episodes in a 24-hour period, the duration (in days) of vomiting and maximum number of vomiting episodes in a 24-hour period, maximum fever, the degree of dehydration (0, 1-5% and 6% or more) and management (nil, outpatient management or hospitalization). For each parameter, 0 to 3 points are given and the maximum score is 20. An AGE episode with a Vesikari score 11 is classified as severe (18,

19). Parents of children with AGE were contacted by phone 1 month after discharge from hospital to collect the data on the course of illness after discharge from hospital required to calculate the Vesikari score.

Sample preparation

Stool samples were prepared as 10% suspensions in PBS (pH 7.4), which were further used for DNA extraction with a QIAamp Stool Mini Kit (Qiagen, Valencia, CA) and molecular detection of enteric bacterial and parasitic pathogens. For the detection of enteric viruses, stool suspensions were centrifuged at $1,600\times g$ for 10 minutes following by $14,000\times g$ for 5 minutes to separate clear supernatant from bacteria and other biological material. This was followed by nucleic acid extraction with an iPrep automatic extractor using a Viral nucleic acid kit (Life Technologies, Foster City, CA). The nucleic acid was used immediately or stored at -80°C until further use.

For NGS application, stool suspensions were vortexed vigorously with zirconia/silica beads and, after centrifugation ($8,600\times g$ for 5 min), supernatants were treated with nucleases to eliminate non-capsulated nucleic acids. For nuclease treatment, 130 μl of supernatant was filtered using a $0.45\text{ }\mu\text{m}$ filter and incubated with 1 μl of RNase A (1 mg/ml) (Life Technologies) for 15 minutes at room temperature. After RNase treatment, 4 μl of Turbo DNase (2 U/ μl) (Life Technologies) and 1 μl of Benzonase (10 kU) (Novagen, San Diego, CA) were added to the reaction and incubated at room temperature for 45 minutes. Viral nucleic acid was extracted from 140 μl of nuclease treated stool suspension using the NucliSENS[®] easyMAG[®] isolation protocol (BioMerieux, Durham, NC). Following extraction, 1 μl of RNase OUT (Life Technologies) was added to each of the extracted nucleic acid samples.

Culturing methods for enteric bacteria

For the detection of the most common bacterial pathogens, stool samples were also inoculated on blood agar plate, xylose lysine deoxycholate (XLD) plates directly and, after enrichment in selenite-F broth, on Karmali plate. Blood agar, XLD plates and selenite F-broth were incubated for 24 hours at 37°C in normal atmosphere and Karmali plate for 48 hours at 37°C in microaerophilic condition. Suspected colonies for specific enteric bacteria on agar plates were further characterized to the species level on a microflex LT MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA).

Molecular testing

Stool samples were screened for the most prevalent bacterial, viral and parasitic agents. The list of agents selected for the testing scheme was prepared on the basis of our previous findings and some publications from European countries reporting gastroenteritis etiology in young children (14, 20). For the detection of the agents, in-house (RT)-qPCR protocols were implemented. In the viral panel, group A rotaviruses (RoV), Adenoviruses species F (AdVF), genogroup I and II noroviruses (NoV-I, NoV-II), human astroviruses (HAstV), human sapoviruses (HSaV), human bocaviruses I-IV (HBoV-I-HBoV-IV), human coronaviruses (HCoV), and parechoviruses (PeV) were tested, using the amplification protocols from the reference publications (21-28). In the bacterial panel, *Campylobacter*

spp., *Salmonella* sp., *Yersinia* sp., *Shigella* sp. and *Clostridium difficile* were included, following already described and published protocols (29, 30). Among the parasites, *Cryptosporidium* spp. and *Giardia duodenalis* were included, also using molecular methods described in previous publications (31, 32).

Viral targets were amplified in a StepOne™ real-time PCR machine (Life Technologies) using AgPath-ID™ One-Step RT-PCR Reagents (Life Technologies) and Platinum® Quantitative PCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA, USA).

For the exclusion of potential asymptomatic carriers of specific viruses, the cycle of threshold (Ct) value distributions for targets with a prevalence of >5% (RoV, AdV-F, NoV-II and PeV) is shown with histograms of the Ct distributions. Since Ct values depend on target concentration, they were also used for comparison of different targets in multiple viral infections. Amplification of *Campylobacter* spp., *Salmonella* sp., *Yersinia* sp., and *Shigella* sp. target genes was performed using a LightCycler® FastStart DNA Master^{PLUS} HybProbe (Roche Applied Science) and LightCycler® 2.0 platform (Roche Applied Science). Detection of *C. difficile* genes encoding toxin A and B, *tcdA* and *tcdB*, was done using TaqMan® Fast Virus 1-Step Master Mix (Life Technologies) and the 7500 Fast Real-Time PCR System (Life Technologies).

For the determination of diarrheagenic groups of *E. coli*, screening for the presence of the genes listed below was performed with mixed bacterial cultures grown on primary blood agar plate. In the case of positive PCR results, at least 15 colonies were sub-cultured from the same primary-culture plate and retested to obtain isolates positive for the tested genes. Up to 10 colonies or an equal volume of mixed microbial culture (approximately one loop of microbial culture (1 µl)) were picked and re-suspended in 200 µl 10% Chelex-100 in 1x TE buffer (Sigma-Aldrich, St. Louis, MO). The suspension was heated for 5 min at 100°C, centrifuged at 2200×g for 5 min and the supernatant was used further for molecular detection of diarrheagenic groups of *E. coli* targeting the *vtx1*, *vtx2* genes of verocytotoxin-producing *E. coli* (VTEC), the *eae* gene of enteropathogenic *E. coli* (EPEC) and some VTEC strains, the *eltA* and *estA* genes of enterotoxigenic *E. coli* (ETEC), the *ipaH* gene of enteroinvasive *E. coli* (EIEC) (33), and the *aat* (AAp), *aggR*, and *aap* genes of enteroaggregative *E. coli* (EAggEC) (34). Detection of O serogroups was performed according to the instructions of antisera producers (Statens Serum Institut; Denmark, Sifin; Germany, Denka Seiken; Japan). All intimin positive *E. coli* isolates with O serogroups of classical and newly recognized EPEC O:H serotypes (35) were interpreted as EPEC (33).

Nucleotide sequence analysis, next generation sequencing (NGS)

Stool samples with no pathogen detected in our screening were included in the metagenomic analysis for the detection of possible new or newly emerging viruses. Extracted nucleic acid, prepared for NGS application, was reverse transcribed using random primers with a Superscript III reverse transcription kit (Life Technologies) and, after RNase H treatment (Life Technologies), the second DNA strand was synthesized with a Klenow fragment (Life Technologies). DNA was purified and used for DNA library preparation (Life Technologies). Purified libraries were loaded onto a 314 IonTorrent chip for sequencing in an IonTorrent

PGM machine (Life Technologies). Sequences obtained in NGS were analyzed using Geneious software (Biomatters Ltd., New Zealand).

Statistical methods

The detection rates of each individual pathogen in AGE and control groups were compared by the chi-square test or Fisher's exact test. The two tests were also used for a comparison of clinical parameters of children with AGE according to the detected pathogens. In order to assess differences of mean Ct values between AGE cases and the control group, we used the Independent Samples t-test for each pathogen. In addition, for each virus the proportion (with 95% confidence interval) of multiple infections in which a specific viral pathogen had a lower viral load than the co-detected viruses was calculated. For all comparisons, a p value <0.05 indicated statistical significance. Statistical analyses were performed using IBM SPSS version 21.

Results

Study population

Consent for inclusion in the study was obtained for 359 children with AGE and for 89 children in the control group. However, stool samples were obtained from 297/359 (82.7%) children with AGE and 88/89 (98.9%) control group participants and these patients were included in the study. The main reason for unsuccessful sampling was that some children with AGE were discharged from hospital after a short period of time, during which they were rehydrated, i.e., prior to defecation and obtaining a specimen. The AGE group and control group differed in the average age (23.3 versus 27.7 months; $p=0.023$) and in the male/female ratio (1.3 (169/128) for patients with AGE and 5.8 (75/13) in the control group; $p<0.001$).

Enrollment of AGE cases was highest from March 2012 – May 2012, when altogether 115 (32.0%) children were included, while enrollment in the control group showed more or less stable pattern throughout the study enrollment period. Of 88 children included in the control group, the questionnaire on the appearance of diarrhea within 7 days after discharge from hospital was returned by 51 participants; none of them reported having diarrhea. The subgroup for which the questionnaire was obtained and the subgroup for which the requested information was not available did not differ significantly in terms of sex and age.

Etiology of gastroenteritis

Using broad range molecular testing, at least one agent was detected in 91.2% of children with AGE and in 27.3% of control group participants (Fig. 1). In Table 1, the detection rate of viral, bacterial, and parasitic agents in stool samples, together with the mean Ct value obtained in (RT)qPCR in AGE and control groups are presented. In both groups, the most prevalent agents were viruses, followed by bacteria and parasites; more than one agent was demonstrated in several children. Among viruses, RoV, NoV-II, AdV, HAdV and PeV were significantly more often detected in the AGE than in the control group, while among bacteria only *Campylobacter* sp. and *Salmonella* sp. showed a detection difference between the study groups. The prevalence of parasites was too low to enable reliable statistical analysis (Table

1). The proportion of mixed “infections” in the AGE group was very high (41.8%, 124/297), with a complex mixture of agents identified. The most prevalent combinations were the presence of more than one virus (in 19.9%) and the combination virus-bacteria (in 19.5%) (Fig. 1). There was no pattern of co-presence in terms of the specific pathogen combination, although PeV and HSAV were the most frequent viral combination.

Nucleic acid sequences of potential viral pathogens were detected in 16 out of 38 tested specimens with NGS analysis (Table 2). In 6 cases, nucleotide sequences of viruses included in but missed by our testing methods were obtained (1x HSAV, 2x PeV, 1x NoV GII.12, 1x NoV GII.4, 1x PeV and NoV GII.4), while in 10 stool specimens viruses not encompassed in the basic testing were demonstrated, including specific enterovirus sequences (4 specimens), human AstV MLB (3 samples), human AdV species C (2 samples) and picobirnavirus-like sequences (1 sample). The last named probably represents a distant variant of a currently published picobirnavirus (36), since only 47% of the deduced amino acid sequence identity was found with a BLASTx search in GenBank (most identical to picobirnavirus TK/MN/2011 found in turkey faeces).

Adding this NGS result to the previous list of detected agents, an overall 97.0% (288/297) detection rate in the AGE group was achieved. However, in many cases, a high Ct value indicated a very low target concentration (Table 1).

The highest prevalence of the majority of viral agents in children with AGE was in the age group of 7-36 months (Fig. 2). However, there were several exceptions: the prevalence of AdV-F was highest in the age group <12 months and declined to undetected in children >36 months old. In contrast, the prevalence of NoV-II was highest in children >60 months of age. The detection peak of RoV was in the age group 24-36 months. For several other viral targets, no specific age-related trend was observed.

Among bacteria, pathogenic *E. coli* (11.7%) and *C. difficile* (8.1%) were the most frequently detected in patients with AGE, but none of them had a significantly higher prevalence in the AGE than in the control group. Within 30 *E. coli* positive samples in children with AGE, EAaggEC was detected in 17, EPEC in 7, VTEC in 4 and ETEC+EAaggEC and EPEC +EAaggEC in 1 sample each. *E. coli* was present as a single pathogen in only 3 out of 30 (10%) positive AGE samples. In addition, the intimin (*eae*) gene was detected in 17 *E. coli* isolates obtained from children with AGE and in 4 isolates obtained from controls; O groups of the isolates did not belong to classical or newly recognized EPEC. Although *C. difficile* was detected in 8.1% (24/295) in the AGE group it was detected as a single pathogen in only 2 out of 24 AGE positive cases. The mean Ct value was 32.2 and also the severity score was not significantly higher in cases with *C. difficile* “(co)infection” in comparison to cases with other pathogens (Table 1 and 3). The median age of children with demonstrated *C. difficile* was 13 months with the highest prevalence in the age group <6 months (18.7%); the prevalence declined with increasing age. Only 2/24 *C. difficile* positive children were older than 24 months. All 5 *C. difficile* positive samples in the control group were from infants <12 months of age.

Standard culturing method for bacteria was less sensitive than molecular methods – the detection rates were 18.5% versus 27.3% in AGE cases and 0 versus 10.2% in the control group. Looking at a specific pathogen, the standard culturing method failed to identify 29 of 30 *E. coli* positive samples, 3 of 14 salmonella positive samples and 1/15 *Campylobacter* positive samples. One *Shigella* positive sample was also missed by the standard method; no *C. difficile* specific molecular diagnostic procedure was used.

Cryptosporidium spp. and *Giardia duodenalis* were detected rarely (Table 1).

Cryptosporidium spp. was found in 8 children with AGE and was the only pathogen in 3 of them. *C. parvum* predominated over other *Cryptosporidium* species (6 and 2 cases, respectively). *C. parvum* was also detected in a child from the control group. In a single *G. duodenalis* positive sample, co-detection with rotaviruses was established.

Clinical evaluation of the results

Comparing AGE and control groups, a significant difference in the prevalence was found only for some of the tested agents (Table 1). In addition, in the stools of patients with AGE, more than one agent was often present and the Ct values for some viruses were fairly high (for example for the majority of PeV and several representatives of other viruses; Table 1 and 4). A significantly lower mean Ct value in the AGE than in the control group was found only for rotavirus but not for other viruses (Table 1).

According to Vesikari score (a measure of the AGE severity), there were no significant differences among pathogens, although RoV, *Salmonella* sp. and *E. coli* cases presented with a slightly higher mean severity score (>13.80) than other pathogens (Table 3). For *Giardia duodenalis*, only one positive case with RoV co-infection was noted with a high Vesikari score (17.00).

Assessment of the individual clinical parameters revealed that *Salmonella* cases more often presented with a higher fever (>39°C) and had longer duration of diarrhoea (>6 days). Children with *Campylobacter* infections were found significantly more frequently in the group without vomiting, which was in contrast to RoV infections (Table 3). The co-presence of more than one agent was not associated with more severe disease, regardless of the type of co-infections (virus-virus, virus-bacteria, ...) or the number of detected pathogens (one to four).

Discussion

Since in many cases it is not possible to differentiate between bacterial and viral enteric infection clinically, a laboratory diagnostic strategy to determine the etiology in a reasonable time is crucial for management of patients. However, no comprehensive study on the etiology of AGE in hospitalized children in Slovenia targeting all three major pathogen groups (viruses, bacteria and parasites) in the diagnostic scheme has been reported.

In the present case-control study, viruses, bacteria and/or parasites were found in stool specimens obtained from children with AGE in 91.2% using broad range molecular testing, and in an additional 5.8% cases using NGS, resulting in diagnosis of 97% cases. NGS is an

important tool in pathogen discovery, since many new or emerging viruses have already been successfully detected with this method (37-42). In our case, NGS provided important information on viral pathogens, which were either not detected with our existing method or represented a potentially new enteric pathogen. It appeared that the molecular test for AstV should be updated additionally to detect at least viral variants such as AstV-MLB, which were found in 3/38 (7.9%) samples screened with NGS. The detection of potential novel picobirnaviruses should be also taken into consideration for further evaluation of their role in the etiology of AGE.

In agreement with previous reports from Europe (13, 43), the present study determined viruses as the most common cause of gastroenteritis among children 0-6 years of age, and RoV was the most frequent virus found. The relatively low proportion of noroviruses, which are a recognized cause of AGE in patients who need hospitalization, was not a surprise because they are mostly found in older children and in adults (44). As shown in countries with a high rotavirus vaccination coverage, such as Austria, Finland and Belgium (45-49), the vaccination effectively prevents severe cases of AGE and drastically decreases the hospitalization rate for diarrhea. Significant reduction of RoV-related and overall AGE incidence may consequently result in higher relative importance of NoV in AGE (9, 10, 46, 50). However, in Slovenia, where RoV vaccine has been available and recommended since 2007, only 26.9% of children were fully vaccinated in 2009, and the proportion was even lower (19.7%) in 2013 (National Institute of Public Health, Slovenia). The vaccination coverage in our country is probably too low for major epidemiological changes in AGE but could be associated with minor changes in RoV epidemiology observed after the introduction of the vaccine (51).

Since the presence of microbes in stool does not confirm their causal association with AGE, we tried to assess their clinical significance with the inclusion of a control group of children without diarrhea, and by measurement of the microorganism's burden in the stool specimen by semi-quantitative PCR assays (low Ct value indicating high burden and vice versa). The latter approach is based on the premise that the chances of a causal relationship are greater with a higher burden than with a lower burden (52). Of 11 viral targets detected in stool specimens, only RoV, NoV-II, AdV, AstV and PeV were significantly more frequent in patients with AGE than in the control group without diarrhea (Table 1). This finding suggests that the role of other viruses is questionable and that they are probably not clinically important pathogens in children <6 years old, hospitalized for AGE. Our results also corroborated previous reports indicating that CoV (53), HSAV and BoV (54, 55) are rarely found in children <6 years old, who are hospitalized for AGE, and suggest that the causal relationship between these and several other viruses detected in the present study and AGE is questionable. The conclusion is further supported by the fairly high Ct values for the viruses and by the finding that a significantly lower mean Ct value in AGE than in the control group was found only for rotavirus but not for other viruses (Table 1). The same is probably valid also for PeV: even though it is ranked the second most common virus demonstrated in stool specimens and was found more often in patients with AGE than in control patients, the low viral burden (comparable to those found in the control group) and very frequent co-presence of other potential etiologic agents of AGE do not reliably support its clinical significance (Table 1 and 4). According to the literature, the role of PeV in AGE

has not been completely elucidated. Some authors have supported an etiologic role of PeV in gastroenteritis, especially for genotypes 1, 2, 4-8 (56), while others have reported the detection of PeV in stool irrespective of clinical presentation (57). NoVI, HSaV, CoV, BoV1, BoV2/4, BoV3, and PeV thus do not need to be included in the routine diagnostic approaches in hospitalized children <6 years old with AGE. However, there might be some differences in the relative frequencies of enteric pathogens in various geographical regions in the world, suggesting the need for appropriate evaluation of the relevant diagnostic list for enteric pathogens. Bacterial pathogens were much less prevalent than viruses in AGE. This finding was expected and agrees with results from Europe, USA and Australia, where bacterial pathogens have been shown to contribute 5-20% of gastroenteritis cases in this age group (43, 58). In the present study, only *C. jejuni/coli* and *Salmonella* sp. were found significantly more often in children with AGE than in control persons, while *E. coli* and *C. difficile* were not. As reported previously, small children are frequently colonized with *C. difficile*; they provide a potential reservoir for toxigenic strains of *C. difficile* and are an important factor in pathogen circulation (59). In the present study, the detection rate was the highest among infants 0-6 months, and was very low in children >36 months old; the age pattern corroborates previous reports (59, 60). Other clues for a dubious etiologic role of *C. difficile* in small children with AGE were the finding that *C. difficile* was detected as the sole agent in the stool specimen in only 2/24 cases and the fact that the *C. difficile* burden was very low and comparable to that found in the control group.

In our study, the most prevalent *E. coli* pathotype was EAggEC (19/30 *E. coli* positive samples in AGE, 2/4 in control group). However, intimin positive samples, not considered to be EPEC, may represent additional pathogenic potential (33). Nevertheless, as with *C. difficile*, the occurrence of *E. coli* was also comparable in patients with AGE and in the control group, and in only 3/30 children with AGE was it the only agent found in a stool specimen (one EPEC O88, one EPEC O26 and one EAggEC strain). Cohen et al (61) suggested that in children, diarrheagenic *E. coli* may represent up to 10% of gastroenteritis cases but may be present in the stool of asymptomatic children and coincide with other viral infections. This conclusion correlates very closely with our results and suggests that the detection of *E. coli* in children should be interpreted with caution and together with other microbiological results and clinical observations.

While parasites are an important cause of childhood diarrhea in the developing world (62), their carriage in stool is low in children living in Europe (63, 64) where especially *Cryptosporidium* spp. and *G. duodenalis* are mostly detected (65). Though *G. duodenalis* is considered to be one of the commonest parasites of humans globally and giardiasis is the most common notifiable protozoan disease in patients in Slovenia (National Institute of Public Health, Slovenia), the course of giardiasis is less acute, which might explain its low prevalence in our study, which encompassed patients with diarrhoea duration of <10 days. The predominance of zoonotic *C. parvum* over other *Cryptosporidium* spp. in human cryptosporidiosis in Slovenia is in agreement with the study conducted by Soba and Logar (66) and might reflect the predominantly rural origin of the patients.

One of the most challenging tasks in the interpretation of gastroenteritis etiology, especially when multi-target methods are applied, is the clinical relevance of co-infections. In our

study, 41.8% of samples obtained from children with AGE contained more than one potentially relevant pathogen, which is slightly more than reported elsewhere (13, 15, 67-69). Previous studies have shown that viral load is of clinical importance for at least some viral pathogens (52, 70-72). In our study, viral pathogens with a clear role in gastroenteritis, such as group A rotaviruses, had much lower Ct values than other viruses, such as PeV, BoV, and HSAV. Although Ct values of different targets cannot be compared directly, large differences in Ct values between co-detected viruses (differences in mean Ct value between targets >7) probably suggest important viral load differences (Table 1). It would be of interest to obtain absolute quantitative data using new quantification methods, such as digital PCR (73).

The concentrations of pathogenic bacteria in stool have not yet been correlated with the pathogenesis or severity of diarrhea, nor have Ct values been evaluated as a measure for distinction between infection and colonization. In the present study, the bacterial burden in stool was substantially lower for *C. difficile* (most probably colonizer) than for *Campylobacter* and *Salmonella* sp. (most probably actual causes of AGE). However, these very preliminary data indicating that low bacterial burden suggests colonization needs further substantiation.

Recently, many new or novel methods with a syndromic approach have been presented and have shown good sensitivity; some of them have already been FDA approved (74, 75). With the implementation of such methods, co-infections will be noted more often. In order to obtain a more balanced delineation of a potential pathogen role in the disease, information on (viral) burden would be helpful and the inclusion of a control group(s) is needed. However, the most powerful combination for accurate diagnostics remains a combination of reliable analytical data in conjunction with careful clinical observations.

A limitation of our study was the relatively low number of controls, which were not precisely matched, resulting in a significant difference in age in sex between cases and controls. The observed boy predominance in the control group was probably due to sex-related differences in elective surgical procedures, such as inguinal hernia repair, which has been shown to be more prevalent in boys than in girls and testicular retention surgery.

In conclusion, our study provides supportive information on the use of a syndromic approach in the diagnostics of acute gastroenteritis for hospitalized children 0-6 years old, with a testing scheme including RoV, NoV-II, AdV, AstV, *Campylobacter* sp. and *Salmonella* sp. Using such an approach, the etiology of AGE was established in 83.5%. We believe that the proposed testing scheme is applicable not only in Slovenia but also in other geographic regions, but the testing algorithm should be evaluated based on epidemiologic investigation for specific region. Molecular multiplex methods are the best choice since they offer rapid, specific and sensitive diagnostics. An important challenge is the co-presence of several agents and the interpretation of such “multiple infections”; the challenge could be at least partially resolved by acquiring quantitative data for viral targets.

Acknowledgement

The authors would like to thank Dr. Mateja Poljšak-Prijatelj, Alenka Andlovic, MD, Irena Šest, Sabina Islamovi, Nataša Krošelj and Jana Šuštar for stool sample processing and performing laboratory diagnostics procedures, and medical staff from the Department of Pediatric Surgery and Intensive Care and from the Department of Infectious diseases for their help in sample collection.

The study was partially supported by the Slovenian Research Agency (ARRS), project numbers J3-4252, J1-5433, P3-0296, P3-0083 and United States National Institutes of Health grant # U54 AI057158.

Literature

1. Liu L, Johnson HL, Cousens S, et al. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *The Lancet*. 2012; 379:2151–2161.
2. Fletcher SM, McLaws ML, Ellis JT. Prevalence of gastrointestinal pathogens in developed and developing countries: systematic review and meta-analysis. *J Public Health Res*. 2013; 2:42–53. [PubMed: 25170480]
3. Lanata CF, Fischer-Walker CL, Olascoaga AC, et al. Global causes of diarrheal disease mortality in children <5 years of age: a systematic review. *PLoS One*. 2013; 8:e72788. [PubMed: 24023773]
4. Das JK, Salam RA, Bhutta ZA. Global burden of childhood diarrhea and interventions. *Curr Opin Infect Dis*. 2014; 27:451–458. [PubMed: 25101554]
5. Payne DC, Boom JA, Staat MA, et al. Effectiveness of pentavalent and monovalent rotavirus vaccines in concurrent use among US children <5 years of age, 2009–2011. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2013; 57:13–20. [PubMed: 23487388]
6. Payne DC, Selvarangan R, Azimi PH, et al. Long-term Consistency in Rotavirus Vaccine Protection: RV5 and RV1 Vaccine Effectiveness in US Children, 2012–2013. *Clin Infect Dis*. 2015; 61:1792–1799. [PubMed: 26449565]
7. Cortese MM, Immergluck LC, Held M, et al. Effectiveness of monovalent and pentavalent rotavirus vaccine. *Pediatrics*. 2013; 132:e25–33. [PubMed: 23776114]
8. Leshem E, Moritz RE, Curns AT, et al. Rotavirus vaccines and health care utilization for diarrhea in the United States (2007–2011). *Pediatrics*. 2014; 134:15–23. [PubMed: 24913793]
9. Koo HL, Neill FH, Estes MK, et al. Noroviruses: The Most Common Pediatric Viral Enteric Pathogen at a Large University Hospital After Introduction of Rotavirus Vaccination. *J Pediatric Infect Dis Soc*. 2013; 2:57–60. [PubMed: 23687584]
10. Payne DC, Vinje J, Szilagyi PG, et al. Norovirus and medically attended gastroenteritis in U.S. children. *The New England journal of medicine*. 2013; 368:1121–1130. [PubMed: 23514289]
11. Smits SL, Schapendonk CM, van Beek J, et al. New viruses in idiopathic human diarrhea cases, the Netherlands. *Emerg Infect Dis*. 2014; 20:1218–1222. [PubMed: 24964003]
12. van Leeuwen M, Williams MM, Koraka P, Simon JH, Smits SL, Osterhaus AD. Human picobirnaviruses identified by molecular screening of diarrhea samples. *J Clin Microbiol*. 2010; 48:1787–1794. [PubMed: 20335418]
13. Amar CF, East CL, Gray J, Iturriza-Gomara M, Maclure EA, McLauchlin J. Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993–1996). *Eur J Clin Microbiol Infect Dis*. 2007; 26:311–323. [PubMed: 17447091]
14. Simpson R, Aliyu S, Iturriza-Gomara M, Desselberger U, Gray J. Infantile viral gastroenteritis: on the way to closing the diagnostic gap. *J Med Virol*. 2003; 70:258–262. [PubMed: 12696113]
15. Onori M, Coltella L, Mancinelli L, et al. Evaluation of a multiplex PCR assay for simultaneous detection of bacterial and viral enteropathogens in stool samples of paediatric patients. *Diagn Microbiol Infect Dis*. 2014; 79:149–154. [PubMed: 24656922]
16. Martinez MA, Soto-Del Rio Mde L, Gutierrez RM, et al. DNA microarray for detection of gastrointestinal viruses. *J Clin Microbiol*. 2015; 53:136–145. [PubMed: 25355758]

17. Wolffs PF, Bruggeman CA, van Well GT, van Loo IH. Replacing traditional diagnostics of fecal viral pathogens by a comprehensive panel of real-time PCRs. *J Clin Microbiol.* 2011; 49:1926–1931. [PubMed: 21430103]
18. Ruuska T, Vesikari T. Rotavirus disease in Finnish children: use of numerical scores for clinical severity of diarrhoeal episodes. *Scand J Infect Dis.* 1990; 22:259–267. [PubMed: 2371542]
19. Vesikari T. Rotavirus vaccination: a concise review. *Clin Microbiol Infect.* 2012; 18(Suppl 5):57–63. [PubMed: 22882248]
20. Iturriza Gomara M, Simpson R, Perault AM, et al. Structured surveillance of infantile gastroenteritis in East Anglia, UK: incidence of infection with common viral gastroenteric pathogens. *Epidemiol Infect.* 2008; 136:23–33. [PubMed: 17313697]
21. Nix WA, Maher K, Johansson ES, et al. Detection of all known parechoviruses by real-time PCR. *J Clin Microbiol.* 2008; 46:2519–2524. [PubMed: 18524969]
22. Kageyama T, Kojima S, Shinohara M, et al. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J Clin Microbiol.* 2003; 41:1548–1557. [PubMed: 12682144]
23. Svraka S, van der Veer B, Duizer E, Dekkers J, Koopmans M, Vennema H. Novel approach for detection of enteric viruses to enable syndrome surveillance of acute viral gastroenteritis. *J Clin Microbiol.* 2009; 47:1674–1679. [PubMed: 19339472]
24. Svraka S, Duizer E, Vennema H, et al. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. *J Clin Microbiol.* 2007; 45:1389–1394. [PubMed: 17360839]
25. Lion T, Baumgartinger R, Watzinger F, et al. Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. *Blood.* 2003; 102:1114–1120. [PubMed: 12702513]
26. Gutierrez-Aguirre I, Steyer A, Boben J, Gruden K, Poljsak-Prijatelj M, Ravnkar M. Sensitive detection of multiple rotavirus genotypes with a single reverse transcription-real-time quantitative PCR assay. *J Clin Microbiol.* 2008; 46:2547–2554. [PubMed: 18524966]
27. Kuypers J, Martin ET, Heugel J, Wright N, Morrow R, Englund JA. Clinical disease in children associated with newly described coronavirus subtypes. *Pediatrics.* 2007; 119:e70–76. [PubMed: 17130280]
28. Lu X, Chittaganpitch M, Olsen SJ, et al. Real-time PCR assays for detection of bocavirus in human specimens. *J Clin Microbiol.* 2006; 44:3231–3235. [PubMed: 16954253]
29. Belanger SD, Boissinot M, Clairoux N, Picard FJ, Bergeron MG. Rapid detection of *Clostridium difficile* in feces by real-time PCR. *J Clin Microbiol.* 2003; 41:730–734. [PubMed: 12574274]
30. Cunningham SA, Sloan LM, Nyre LM, Vetter EA, Mandrekar J, Patel R. Three-hour molecular detection of *Campylobacter*, *Salmonella*, *Yersinia*, and *Shigella* species in feces with accuracy as high as that of culture. *J Clin Microbiol.* 2010; 48:2929–2933. [PubMed: 20519461]
31. Jothikumar N, da Silva AJ, Moura I, Qvarnstrom Y, Hill VR. Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays. *J Med Microbiol.* 2008; 57:1099–1105. [PubMed: 18719179]
32. Verweij JJ, Schinkel J, Laeijendecker D, van Rooyen MA, van Lieshout L, Polderman AM. Real-time PCR for the detection of *Giardia lamblia*. *Mol Cell Probes.* 2003; 17:223–225. [PubMed: 14580396]
33. Persson S, Olsen KE, Scheutz F, Krogfelt KA, Gerner-Smidt P. A method for fast and simple detection of major diarrhoeagenic *Escherichia coli* in the routine diagnostic laboratory. *Clin Microbiol Infect.* 2007; 13:516–524. [PubMed: 17331124]
34. Cerna JF, Nataro JP, Estrada-Garcia T. Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. *J Clin Microbiol.* 2003; 41:2138–2140. [PubMed: 12734261]
35. Scheutz F, Strockbine N, Genus I. *Escherichia*. In: Garrity, GM., editor. *Bergey's Manual of Systematic Bacteriology* ed. Springer; US: 2005. p. 607-624.
36. Verma H, Mor SK, Erber J, Goyal SM. Prevalence and complete genome characterization of turkey picobirnaviruses. *Infect Genet Evol.* 2015; 30:134–139. [PubMed: 25530436]
37. Chiu CY. Viral pathogen discovery. *Curr Opin Microbiol.* 2013; 16:468–478. [PubMed: 23725672]

38. Phan TG, Mori D, Deng X, et al. Small circular single stranded DNA viral genomes in unexplained cases of human encephalitis, diarrhea, and in untreated sewage. *Virology*. 2015; 482:98–104. [PubMed: 25839169]
39. Phan TG, Nordgren J, Ouermi D, et al. New astrovirus in human feces from Burkina Faso. *J Clin Virol*. 2014; 60:161–164. [PubMed: 24775733]
40. Delwart E. A roadmap to the human virome. *PLoS Pathog*. 2013; 9:e1003146. [PubMed: 23457428]
41. Li L, Deng X, Mee ET, et al. Comparing viral metagenomics methods using a highly multiplexed human viral pathogens reagent. *J Virol Methods*. 2015; 213:139–146. [PubMed: 25497414]
42. Ng TF, Kondov NO, Deng X, Van Eenennaam A, Neibergs HL, Delwart E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. *J Virol*. 2015; 89:5340–5349. [PubMed: 25740998]
43. Lorrot M, Bon F, El Hajje MJ, et al. Epidemiology and clinical features of gastroenteritis in hospitalised children: prospective survey during a 2-year period in a Parisian hospital, France. *Eur J Clin Microbiol Infect Dis*. 2011; 30:361–368. [PubMed: 21128089]
44. Franck KT, Fonager J, Ersboll AK, Bottiger B. Norovirus epidemiology in community and health care settings and association with patient age, Denmark. *Emerg Infect Dis*. 2014; 20:1123–1131. [PubMed: 24960024]
45. Paulke-Korinek M, Kollaritsch H, Aberle SW, et al. Sustained low hospitalization rates after four years of rotavirus mass vaccination in Austria. *Vaccine*. 2013; 31:2686–2691. [PubMed: 23597718]
46. Hemming M, Rasanen S, Huhti L, Paloniemi M, Salminen M, Vesikari T. Major reduction of rotavirus, but not norovirus, gastroenteritis in children seen in hospital after the introduction of RotaTeq vaccine into the National Immunization Programme in Finland. *Eur J Pediatr*. 2013; 172:739–746. [PubMed: 23361964]
47. Standaert B, Gomez JA, Raes M, Debrus S, Velazquez FR, Postma MJ. Impact of rotavirus vaccination on hospitalisations in Belgium: comparing model predictions with observed data. *PLoS one*. 2013; 8:e53864. [PubMed: 23349754]
48. Parez N, Giaquinto C, Du Roure C, et al. Rotavirus vaccination in Europe: drivers and barriers. *Lancet Infect Dis*. 2014; 14:416–425. [PubMed: 24758998]
49. Braeckman T, Van Herck K, Meyer N, et al. Effectiveness of rotavirus vaccination in prevention of hospital admissions for rotavirus gastroenteritis among young children in Belgium: case-control study. *BMJ*. 2012; 345:e4752. [PubMed: 22875947]
50. Bucardo F, Reyes Y, Svensson L, Nordgren J. Predominance of norovirus and sapovirus in Nicaragua after implementation of universal rotavirus vaccination. *PLoS One*. 2014; 9:e98201. [PubMed: 24849288]
51. Steyer A, Sagadin M, Kolenc M, Poljsak-Prijatelj M. Molecular characterization of rotavirus strains from pre- and post-vaccination periods in a country with low vaccination coverage: the case of Slovenia. *Infect Genet Evol*. 2014; 28:413–425. [PubMed: 24999235]
52. Platts-Mills JA, Liu J, Hout ER. New concepts in diagnostics for infectious diarrhea. *Mucosal Immunol*. 2013; 6:876–885. [PubMed: 23881355]
53. Jevnik M, Steyer A, Zrim T, et al. Detection of human coronaviruses in simultaneously collected stool samples and nasopharyngeal swabs from hospitalized children with acute gastroenteritis. *Virol J*. 2013; 10:46. [PubMed: 23379823]
54. Rovida F, Campanini G, Piralla A, Adzasehoun KM, Sarasini A, Baldanti F. Molecular detection of gastrointestinal viral infections in hospitalized patients. *Diagn Microbiol Infect Dis*. 2013; 77:231–235. [PubMed: 24035383]
55. Medici MC, Tummolo F, Albonetti V, Abelli LA, Chezzi C, Calderaro A. Molecular detection and epidemiology of astrovirus, bocavirus, and sapovirus in Italian children admitted to hospital with acute gastroenteritis, 2008–2009. *J Med Virol*. 2012; 84:643–650. [PubMed: 22337304]
56. Pajkrt D, Benschop KS, Westerhuis B, Molenkamp R, Spanjerberg L, Wolthers KC. Clinical characteristics of human parechoviruses 4–6 infections in young children. *Pediatr Infect Dis J*. 2009; 28:1008–1010. [PubMed: 19536040]

57. Tapia G, Cinek O, Witso E, et al. Longitudinal observation of parechovirus in stool samples from Norwegian infants. *J Med Virol.* 2008; 80:1835–1842. [PubMed: 18712841]
58. Elliott EJ. Acute gastroenteritis in children. *BMJ.* 2007; 334:35–40. [PubMed: 17204802]
59. Rousseau C, Poilane I, De Pontual L, Maherault AC, Le Monnier A, Collignon A. Clostridium difficile carriage in healthy infants in the community: a potential reservoir for pathogenic strains. *Clin Infect Dis.* 2012; 55:1209–1215. [PubMed: 22843784]
60. Sandora TJ, Fung M, Flaherty K, et al. Epidemiology and risk factors for Clostridium difficile infection in children. *Pediatr Infect Dis J.* 2011; 30:580–584. [PubMed: 21233782]
61. Cohen MB, Nataro JP, Bernstein DI, Hawkins J, Roberts N, Staat MA. Prevalence of diarrheagenic Escherichia coli in acute childhood enteritis: a prospective controlled study. *J Pediatr.* 2005; 146:54–61. [PubMed: 15644823]
62. Kotloff KL, Nataro JP, Blackwelder WC, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet.* 2013; 382:209–222. [PubMed: 23680352]
63. Davies AP, Campbell B, Evans MR, Bone A, Roche A, Chalmers RM. Asymptomatic carriage of protozoan parasites in children in day care centers in the United kingdom. *Pediatr Infect Dis J.* 2009; 28:838–840. [PubMed: 19684527]
64. Vandenberg O, Robberecht F, Dauby N, et al. Management of a Cryptosporidium hominis outbreak in a day-care center. *Pediatr Infect Dis J.* 2012; 31:10–15. [PubMed: 22094626]
65. Guarino A, Ashkenazi S, Gendrel D, Lo Vecchio A, Shamir R, Szajewska H. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition/European Society for Pediatric Infectious Diseases evidence-based guidelines for the management of acute gastroenteritis in children in Europe: update 2014. *J Pediatr Gastroenterol Nutr.* 2014; 59:132–152. [PubMed: 24739189]
66. Soba B, Logar J. Genetic classification of Cryptosporidium isolates from humans and calves in Slovenia. *Parasitology.* 2008; 135:1263–1270. [PubMed: 18664309]
67. Cunliffe NA, Booth JA, Elliot C, et al. Healthcare-associated viral gastroenteritis among children in a large pediatric hospital, United Kingdom. *Emerg Infect Dis.* 2010; 16:55–62. [PubMed: 20031043]
68. Chen SY, Tsai CN, Chao HC, et al. Acute gastroenteritis caused by multiple enteric pathogens in children. *Epidemiol Infect.* 2009; 137:932–935. [PubMed: 19046476]
69. Iturriza-Gomara M, Elliot AJ, Dockery C, Fleming DM, Gray JJ. Structured surveillance of infectious intestinal disease in pre-school children in the community: ‘The Nappy Study’. *Epidemiol Infect.* 2009; 137:922–931. [PubMed: 19017426]
70. Phillips G, Lopman B, Tam CC, Iturriza-Gomara M, Brown D, Gray J. Diagnosing norovirus-associated infectious intestinal disease using viral load. *BMC Infect Dis.* 2009; 9:63. [PubMed: 19442278]
71. Mukhopadhyay I, Sarkar R, Menon VK, et al. Rotavirus shedding in symptomatic and asymptomatic children using reverse transcription-quantitative PCR. *J Med Virol.* 2013; 85:1661–1668. [PubMed: 23775335]
72. Bennett A, Bar-Zeev N, Jere KC, et al. Determination of a Viral Load Threshold To Distinguish Symptomatic versus Asymptomatic Rotavirus Infection in a High-Disease-Burden African Population. *J Clin Microbiol.* 2015; 53:1951–1954. [PubMed: 25854480]
73. Sedlak RH, Jerome KR. Viral diagnostics in the era of digital polymerase chain reaction. *Diagn Microbiol Infect Dis.* 2013; 75:1–4. [PubMed: 23182074]
74. Goldenberg SD, Bacelar M, Brazier P, Bisnauthsing K, Edgeworth JD. A cost benefit analysis of the Luminex xTAG Gastrointestinal Pathogen Panel for detection of infectious gastroenteritis in hospitalised patients. *J Infect.* 2015; 70:504–511. [PubMed: 25449904]
75. Buss SN, Leber A, Chapin K, et al. Multicenter evaluation of the BioFire FilmArray gastrointestinal panel for etiologic diagnosis of infectious gastroenteritis. *J Clin Microbiol.* 2015; 53:915–925. [PubMed: 25588652]

Figure 1.
Prevalence of viral, bacterial and parasitic infections in cases with acute gastroenteritis (AGE) group and control group.

Figure 2.

Age distributed proportion of positive samples for specific viral and bacterial agents in children with acute gastroenteritis.

RoV – rotaviruses; AdV-F – adenoviruses species F; NoV-II – noroviruses genogroup II; AstV – astroviruses; PeV - parechoviruses

Table 1

Detection rate of viral, bacterial and parasitic pathogens in stool samples with the mean Ct value obtained in (RT-)qPCR.

	AGE group			control group			χ^2 or Fisher exact* test
	Prevalence N (%)	95% CI	Ct-mean (S.D.)	Prevalence N (%)	95% CI	Ct-mean (S.D.)	
VIRUSES	245/297 (82.5)	78-87		14/88 (15.9)	8-24		p<0.001
RoV	165/297 (55.6)	50- 61	18.24 (5.2)	1/88 (1.1)	0 - 3	37.7	p<0.001
AdV	22/297 (7.4)	15 - 24	16.41 (9.5)	0/88 (0)	/	/	p<0.001
AstV	16/297 (5.4)	3- 8	17.65 (9.1)	0/88 (0)	/	/	p=0.028
NoVII	44/297 (14.8)	11 - 19	23.63 (6.3)	3/88 (3.4)	0 - 7	24.8 (4.0)	p=0.004
NoVI	9/297 (3.0)	1 - 5	28.14 (8.7)	1/88 (1.1)	0 - 3	31.0	p=0.468
HSaV	13/297 (4.4)	2 - 7	29.06 (8.0)	4/88 (4.5)	0 - 9	29.2 (8.5)	p=1.000
CoV	2/297 (0.7)	0 - 2	36.27 (3.1)	0/88 (0)	/	/	p=1.000
BoV1	7/297 (2.4)	1 - 4	30.88 (6.7)	2/88 (2.3)	0 - 5	33.7 (1.8)	p=1.000
BoV2/4	7/297 (2.4)	1 - 4	28.81 (8.9)	2/88 (2.2)	0 - 5	38.0 (1.1)	p=1.000
BoV3	2/297 (0.7)	0 - 2	35.42 (2.0)	0/88 (0)	/	/	p=1.000
PeV	48/297 (16.2)	12- 20	33.25 (5.5)	3/88 (3.4)	0 - 7	28.5 (3.9)	p=0.002
BACTERIA	81/295 (27.3)	22-33		9/88 (10.2)	4-17		p<0.001
<i>Escherichia coli</i> **	30/257*** (11.7)	8 - 16	n.d.	4/73*** (5.5)	0 - 11	n.d.	p=1.000
<i>Campylobacter jejuni/coli</i>	15/295 (5.1)	3 - 8	28.96 (4.5)	0/88 (0)	/	/	p=0.031
<i>Salmonella sp.</i>	14/295 (4.7)	2 - 7	27.02 (6.8)	0/88 (0)	/	/	p=0.037
<i>Yersinia sp.</i>	0/295 (0)	/	/	0/88 (0)	/	/	/
<i>Shigella sp.</i>	1/295 (0.3)	0 - 1	23.18	0/88 (0)	/	/	p=1.000
<i>Clostridium difficile</i>	24/295 (8.1)	5 - 11	32.18 (4.7)	5/88 (5.7)	1 - 11	32.8 (4.2)	p=0.480
PARASITES	9/295 (3.0)	1-5		1/88 (1.1)	0-3		p=0.329
<i>Cryptosporidium sp.</i>	8/295 (2.7)	1 - 5	26.56 (2.4)	1/88 (1.1)	0 - 3	28.9	p=0.468
<i>Giardia duodenalis</i>	1/295 (0.3)	0 - 1	31.0	0/88 (0)	/	/	p=1.000

n.d. – no data

* χ^2 or Fisher's exact tests were used for the comparison of specific pathogen prevalence between AGE and control groups; statistically significant findings are shown in bold

** In the present study, diarrhoeagenic groups of *E. coli* were detected (VTEC, ETEC, EAaggEC, EPEC, EIEC) by molecular methods, as described in materials and methods

*** *E. coli* molecular test was not performed for 40 AGE cases and 15 control group individuals due to stool sample limitation

Table 2

Detected targets in next generation sequencing analysis of 16 samples, recognized as negative with our in-house testing approach.

Sample	Potential AGE pathogen found	The highest nt (aa) identity in GenBank/ accession number	N of virus-specific contigs	Maximum length of contigs (nt)
1	Sapovirus, genogroup V	97% (99%)/AB924385	3	352
2	Parechovirus A, HPeV-1	93% (94%)/FM242866	55	895
3	Parechovirus A, HPeV-1	92% (96%)/EU024633	62	505
4	Parechovirus A, HPeV-1	90%* (69%)/EU024632	2	198
	Norovirus, genotype GII.4	99% (99%)/KJ685415	288	3223
5	Norovirus, gnotype GII.4	99% (99%)/AB212266	2	476
6	Norovirus, genotype GII.12	97% (99%)/LN854570	245	4510
7	Enterovirus B (Coxsackievirus B5)	88% (90%)/X67706	20	254
8	Enterovirus A, (Coxsackievirus A8)	98% (99%)/KP765687	14	456
9	Enterovirus B (echovirus 6)	88% (98%*)/JQ729993	968	1106
10	Enterovirus B (echovirus 11)	83% (93%)/AJ577589	71	3195
11	Picobirnavirus	74%* (47%)/AHZ46149	19	669
12	Mastrovirus 6 (AstV MLB-2)	98% (99%)/AB829252	12	267
13	Mastrovirus 6 (AstV MLB-2)	99% (99%)/JF742759	>450	6055
14	Mastrovirus 6 (AstV MLB-2)	98% (93%)/AB829252	3	200
15	Human mastadenovirus C	99% (100%)/KF429754	236	2186
16	Human mastadenovirus C	99% (99%)/KF268130	440	4878

* low query coverage

** parechovirus and norovirus sequences were detected in the same sample; nt – nucleotide; aa – aminoacid; AGE – acute gastroenteritis; HPeV – human parechovirus; AstV – astrovirus

Table 3

Vesikari score and specific clinical parameters for agents detected in the group with acute gastroenteritis.

	Vesikari score			χ^2 or Fisher exact test for specific clinical parameters*				
	Mean	S.D.	Range (min-max)	Body temperature ^a	Diarrhoea duration ^b	Defecation frequency ^c	Vomiting duration ^d	Vomiting frequency ^e
VIRUSES								
RoV	13.88	2.30	7-19	ns	ns	ns	less frequently in Vd0 (p<0.001)	less frequently in Vf0 (p<0.001)
AdV-F	12.30	2.11	9-16	more frequently in T1 (p=0.016)	ns	ns	ns	ns
AsV	13.38	2.31	10-17	ns	ns	ns	ns	ns
NoV-I	13.33	1.41	11-15	ns	ns	ns	ns	ns
NoV-II	12.79	2.25	6-18	more frequently in T1 (p=0.005)	ns	ns	ns	ns
HSaV	11.83	2.66	6-17	ns	more frequently in Dd1 (p=0.03)	more frequently in Df1 (p=0.04)	ns	ns
PeV	13.79	2.32	9-17	ns	ns	ns	ns	ns
BACTERIA								
<i>Escherichia</i>	13.86	2.42	9-19	ns	ns	ns	ns	ns
<i>Campylobacter</i> sp.	11.57	2.68	7-17	ns	ns	ns	more frequently in Vd0 (p<0.001)	more frequently in Vf0 (p<0.001)
<i>Salmonella</i> sp.	13.92	3.06	7-18	more frequently in T3 (p=0.001)	more frequently in Dd3 (p=0.02)	ns	ns	ns
<i>Clostridium difficile</i>	13.05	2.10	9-18	ns	ns	ns	ns	ns
PARASITES								
<i>Cryptosporidium</i> sp.	12.57	2.44	10-17	ns	ns	ns	ns	ns
<i>Giardia</i> sp.	17.00	/	17	ns	ns	ns	ns	ns
Monoinfection	13.4	2.54	7-19	ns	ns	ns	ns	ns
Mixed infection	12.4	2.40	7-17	ns	ns	ns	ns	ns

ns – not significant

^abody temperature groups: T1: <38.4 °C; T2: 38.5 °C – 38.9 °C; T3: >39°C

^bdiarrhoea duration groups: Dd1: 1–4 days; Dd2: 5 days; Dd3: 6 days

^cdefecation frequency groups: Df1: 1–3×/day; Df2: 4–5×/day; Df3: 6×/day

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

^d vomiting duration groups: Vd0: vomiting absent; Vd1: 1 day; Vd2: 2 days; Vd3: 3 days
^e vomiting frequency groups: Vf0: vomiting absent; Vf1: 1×/day; Vf2: 2-4×/day; Vf3: 5×/day

Table 4

Proportion (with 95% CI) of multiple “infections” in which a specific viral pathogen was found with a lower viral load (higher Ct value) than the co-detected viruses. In some samples, the presence of >2 viruses was established.

Virus	Proportion	95% CI
PeV	50.6% (41/81)	39.0% - 62.0%
HSaV	13.6% (11/81)	6.1% - 21.1%
AstV	11.1% (9/81)	4.3% - 17.9%
BoV1	7.4% (6/81)	1.7% - 13.1%
RoV	7.4% (6/81)	1.7% - 13.1%
NoVI	6.2% (5/81)	2.7% - 13.6%
BoV2/4	4.9% (4/81)	1.9% - 12%
NoVII	3.7% (3/81)	1.3% - 10.3%
AdVF	3.7% (3/81)	1.3% - 10.3%
BoV3	2.5% (2/81)	0.7% - 8.6%
CoV	1.2% (1/81)	0.2% - 6.7%

PeV – parechoviruses; HsaV – human sapoviruses; AstV – astroviruses; BoV1 – bokavirus genotype 1; BoV2/4 – bokaviruses genotypes 2 and 4; BoV3 – bokavirus genotype 3; RoV – rotaviruses; NoVI – genogroup I noroviruses; NoVII – genogroup II noroviruses; AdVF – adenoviruses species F; CoV - coronaviruses