Detection of potential enteric pathogens in children with severe acute gastroenteritis using the filmarray: Results from a three - years hospital-based survey in Northern Italy

Diagnostic Microbiology & Infectious Disease

DETECTION OF POTENTIAL ENTERIC PATHOGENS IN CHILDREN WITH SEVERE ACUTE GASTROENTERITIS USING THE FILMARRAY: RESULTS FROM A THREE-YEARS HOSPITAL-BASED SURVEY IN NORTHERN ITALY

--Manuscript Draft--

Manuscript Number:	
Article Type:	Original article
Keywords:	Acute gastroenteritis, childcare setting, multiplex PCR, filmarray, infectious disease control, rapid diagnostic assay.
Corresponding Author:	Flora De Conto University of Parma: Universita degli Studi di Parma Parma, ITALY
First Author:	Flora De Conto
Order of Authors:	Flora De Conto
	SHARON DI STEFANO
	MIRKO BUTTRINI
	CLARA MACCARI
	MARIA CRISTINA ARCANGELETTI
	CARLO CHEZZI
	ADRIANA CALDERARO
Abstract:	Acute gastroenteritis (AGE) are leading causes of morbidity and mortality in children. To control AGE, rapid identification of enteric pathogens is needed. The AGE aetiology was investigated from 2018 to 2020 in 2,066 children in Parma (Italy), using the filmarray. Pathogens were detected in 1,101 (53.3%) stool samples; 796 (72.3%) were single infections and 305 (27.7%) mixed infections (72.3% vs. 27.7%, P<0.0001). The highest infection incidence (68.2%) was assessed in children aged 0-5 years. Enteropathogenic Escherichia coli (EPEC) was the most frequently identified pathogen (21.14%), followed by Clostridioides difficile (11.5%), Norovirus (11.44%), and Campylobacter (10.17%). EPEC, Campylobacter, enteroaggregative Escherichia coli, Norovirus, and Rotavirus showed seasonality. The pathogen detection rate decreased between 2018 and 2020 (41.1% vs. 22%, P<0.0001), because of the preventive measures imposed by the severe acute respiratory syndrome coronavirus 2 pandemic. A putative aetiology in half the children examined and an estimate of enteric pathogens epidemiology were assessed.
Suggested Reviewers:	VITO MARTELLA Universita degli Studi di Bari Dipartimento di Medicina Veterinaria vito.martella@uniba.it
	ELEONORA PONTERIO Istituto Superiore di Sanità: Istituto Superiore Di Sanita eleonoraponterio@yahoo.it
	SARAH N. BUSS University of Nebraska Medical Center sarah.buss@wyo.gov
	MELISSA O'NEAL University of South Carolina College of Pharmacy mponeal@email.sc.edu
	JEREMY MEYER Geneva University Hospitals: Hopitaux Universitaires Geneve jeremy.meyer@hcuge.ch

Highlights

- FAGP assessed the putative aetiology in Italian children with AGE from 2018 to 2020
- Bacteria were the most common enteric pathogens and single infections predominated
- The detection rate of enteric pathogens was highest in young children (0-5 years)
- Infection incidence showed seasonal variations for some enteric pathogens
- Preventive measures against SARS-CoV-2 pandemic decreased pathogens detection

DETECTION OF POTENTIAL ENTERIC PATHOGENS IN CHILDREN WITH SEVERE ACUTE GASTROENTERITIS USING THE FILMARRAY: RESULTS FROM A THREE-YEARS HOSPITAL-BASED SURVEY IN NORTHERN ITALY

Flora De Conto*, Sharon Di Stefano, Mirko Buttrini, Clara Maccari, Maria Cristina Arcangeletti, Carlo Chezzi, Adriana Calderaro

Department of Medicine and Surgery, University of Parma, Viale Antonio Gramsci 14, 43126 Parma, Italy

* Author for correspondence:

E-mail: flora.deconto@unipr.it fax: +39 0521 993620

Running title: AGE diagnosis in children in Italy

Conflict of Interest

Adriana Calderaro declares that she is an Editorial Member of *Diagnostic Microbiology and Infectious Disease.*

ABSTRACT

Acute gastroenteritis (AGE) are leading causes of morbidity and mortality in children. To control AGE, rapid identification of enteric pathogens is needed.

The AGE aetiology was investigated from 2018 to 2020 in 2,066 children in Parma (Italy), using the filmarray. Pathogens were detected in 1,101 (53.3%) stool samples; 796 (72.3%) were single infections and 305 (27.7%) mixed infections (72.3% vs. 27.7%, $P<0.0001$). The highest infection incidence (68.2%) was assessed in children aged 0-5 years.

Enteropathogenic *Escherichia coli* (EPEC) was the most frequently identified pathogen (21.14%), followed by *Clostridioides difficile* (11.5%), Norovirus (11.44%), and *Campylobacter* (10.17%). EPEC, *Campylobacter*, enteroaggregative *Escherichia coli*, Norovirus, and Rotavirus showed seasonality. The pathogen detection rate decreased between 2018 and 2020 (41.1% vs. 22%, $P<0.0001$), because of the preventive measures imposed by the severe acute respiratory syndrome coronavirus 2 pandemic. A putative aetiology in half the children examined and an estimate of enteric pathogens epidemiology were assessed.

ABBREVIATIONS

AdV: adenovirus

AGE: acute gastroenteritis

EAEC: enteroaggregative *Escherichia coli*

EIEC: *Shigella*/enteroinvasive *Escherichia coli*

EPEC: enteropathogenic *Escherichia coli*

ETEC: enterotoxigenic *Escherichia coli*

FAGP: FilmArray Gastrointestinal panel

GDH: glutamate dehydrogenase enzyme

IC: immunochromatographic assay

LAMP: Loop-mediated isothermal amplification

NoV: norovirus

PCR: polymerase-chain reaction

RV: rotavirus

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

SaV: sapovirus

STEC: *Shiga*-like toxin-producing *Escherichia coli*

KEYWORDS

Acute gastroenteritis, childcare setting, multiplex PCR, filmarray, infectious disease control, rapid diagnostic assay.

1. INTRODUCTION

Acute gastroenteritis (AGE) are a leading cause of childhood morbidity and mortality with relevant costs for societies. AGE with diarrhea and/or vomiting can last several days and be associated with symptoms such as abdominal pain, cramping, fever, malaise, and anorexia. There are nearly 1.7 billion cases of childhood diarrheal disease worldwide every year, killing about 525,000 children [1].

The etiologic agents of childhood diarrheal disease include bacteria, parasites, and viruses [2]. Age, social, and geographic factors modulate the incidence of enteric pathogens [3].

Most cases of childhood diarrheal disease are due to viral infections principally caused by Rotavirus and Norovirus [4,5]. Despite the vaccine, Rotavirus still causes hospitalization [6]. Bacteria may cause severe diarrhea (bloody stools), and high fever, needing hospital management. Moreover, some enteric bacteria may spread systematically [7]. With enhanced globalization and immigration, clinicians of developed countries diagnose several cases of diarrhea due to protozoa not habitually circulating [7].

Although the epidemiological/clinical evaluation of AGE not always allows differentiating among bacterial, viral, and parasitological etiology, it guides the laboratory diagnosis. Prompt diagnosis optimizes the patient treatment, avoiding incorrect antibiotic use and reducing hospitalization. Conventional methods are laborious, time-consuming, and costly. Multiplex molecular assays considerably reduce the time to result, detecting a greater number of pathogens [8].

This large-scale hospital-based survey in Parma (Northern Italy) from 2018 to 2020 aims to assess the epidemiology of AGE pathogens in children with a multiplex polymerase chain reaction (PCR)-based platform. Moreover, the impact of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic on AGE dynamics was investigated.

2. MATERIALS AND METHODS

2.1 Study setting

This study was performed at the Microbiology and Virology Units of the University-Hospital of Parma, a 1,044-bed tertiary care center with more than 50,000 admissions per year.

The inclusion criteria were subjects ≤14 years old and occurrence of AGE symptoms (diarrhea, vomiting, abdominal pain, and fever).

From January 2, 2018 to December 31, 2020, 2,066 stool samples from as many children were submitted to the laboratory for diagnostic purposes. Table 1 shows the demographic characteristics of the examined children, whose age ranged from ≥13 days to 14 years and 3 months.

Laboratory diagnosis was performed upon medical order. The results were reported in the clinical records of the patients as answer to a clinical suspicion. Patients' identity and medical information were protected and remained anonymous.

2.2 Molecular assays

For the FilmArray gastrointestinal panel assay (FAGP, BioFire Diagnostics-bioMérieux, Italy), 200 µl of each stool sample in Cary-Blair transport medium (Faecal swab; Copan, Italy) were used. The FAGP is a multiplexed PCR, revealing 22 pathogens: *Campylobacter* (*C. jejuni*, *C. coli*, and *C. upsaliensis*), *Clostridioides difficile* (toxin A/B), *Plesiomonas shigelloides*, *Salmonella*, *Yersinia enterocolitica*, *Vibrio* (*V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, with specific detection of *V. cholerae*), enteroaggregative *Escherichia coli* (EAEC), enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC), *Shiga*-like toxin-producing *Escherichia coli* (STEC, with specific detection of *E. coli* O157), *Shigella*/enteroinvasive *Escherichia coli* (EIEC), *Cryptosporidium spp.*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Giardia lamblia*, adenovirus (AdV) F40/41, astrovirus, norovirus (NoV) genogroup GI/GII, rotavirus (RV) group A, and sapovirus (SaV) genogroups I, II, IV, and V. The assay lasting nearly 1 hour was performed in the FilmArray 2.0 platform.

In case of *Clostridioides difficile* detection by FAGP for a sample with a medical prescription requiring this testing, a qualitative assay (Illumigen TM *C.difficile*, Meridian Bioscience, USA), based on the Loop-mediated isothermal amplification (LAMP), was performed [9].

For viral diagnostic purpose, when an adequate aliquot of sample was available (2,023 cases, 97.9%), an Enterovirus-targeting real-time PCR (Enterovirus Q–PCR Alert kit, ElitechGroup, Italy) was performed.

2.3 Conventional diagnostics methods

In case of bacteria detection by FAGP, the sample was submitted to conventional methods for isolation and/or identification purposes (data not shown) [10]. In case of *Clostridioides difficile* revealing by FAGP and LAMP, the detection of glutamate dehydrogenase enzyme (GDH) and toxins A/B by immunocromatographic assay (IC, *C.difficile* Quick check complete, Techlab, USA) as well as culture were performed [9].

For virus detection, electron microscopy was also performed (data not included) [10].

In case of parasites detection by FAGP, the sample was submitted to microscopic examination, *Giardia intestinalis*/*Cryptosporidium* IC, immunofluorescence, and cultures (data not shown) [10-13].

2.4 Statistical analysis

The chi-square test was performed by GraphPad Prism software. $P<0.05$ was considered statistically significant.

3. RESULTS

3.1 Prevalence of enteric pathogens in the study population

A total of 2,066 samples were analyzed, as described in the Methods section. AGE was most common in pediatrics aged 0-5 years (65.3%) and in males than in females, with a ratio of 10:8 (Table 1). The rate of hospitalized children was 62.7%, showing in 2020 a decrease of -21.5% vs. 2019 and of -33.8% vs. 2018.

The FAGP identified at least one etiologic agent in 1,101 samples (53.3%). Figure 1 shows the positivity rates of the pathogens detected; EPEC was the most frequent (21.14%), followed by *Clostridioides difficile* (11.5%), NoV (11.44%), and *Campylobacter* (10.17%). *Cyclospora cayetanensis, Vibrio cholerae* and *Entamoeba histolytica* were not detected.

A total of 1,495 pathogens was revealed. Of these, 949 (63.5%) were bacteria, 476 (31.8%) viruses, and 70 (4.7%) parasites. Specifically, 615 pathogens (41.1%) were revealed in 2018, 551 (36.9%) in 2019, and 329 (22%) in 2020 (41.1% vs. 22%, $P<0.0001$, and 36.9% vs. 22%, $P<0.0001$).

Single infections were 796 (72.3%) and mixed infections 305 (27.7%) (72.3% vs. 27.7%; $P<0.0001$).

Co-infections with two pathogens were detected in 227 (20.6%) of the 1,101 positive samples, compared to 66 (6%) with three and 12 (1.1%) with more than three. The percentage difference in the number of co-infections with two (20.6%) and more than three pathogens (1.1%) was significant ($P<0.0001$). Overall, 147 different co-infections combinations were found (Table 2). The most common included either bacteria (*Campylobacter*, 7.8%, and *Clostridioides difficile*, 6.2%) or NoV (5.2%) in association with EPEC.

Of the 172 samples positive for *Clostridioides difficile* by FAGP, 13 samples (7.6%) belonging to children >2 years old hospitalized in oncologic wards had a medical order requiring *Clostridioides difficile* testing. Of these, 12 cases (92.3%) resulted positive by LAMP. Among these, GDH was revealed in 11 cases (91.7%) and toxin A/B in 3 cases (25%). Finally, in 4 cases (33%) *Clostridioides difficile* strains were isolated.

The RT-PCR assay for Enterovirus gave positive results in 156 (7.7%) of the examined samples; of those, 63 (40.4%) were single infections.

3.2 Age distribution of enteric pathogens

Concerning the distribution of positive samples by age, 68.2% (751/1,101) were from children aged 0-5 years, 19.8% (218/1,101) from children aged 6-10 years, and 12% (135/1,101) from children aged 11-14 years. Of the 1,495 pathogens revealed, 1,051 (70.4%) were detected in children aged 0-5 years, 273 (18.2%) in children aged 6-10 years, and 171 (11.4%) in children aged 11-14 years (70.4% vs. 11.4%, $P<0.0001$). Of the pathogens revealed in children aged 0-5 years, 58.5% were bacteria, 38% viruses, and 3.5% parasites.

Concerning the distribution of co-infections by age, 226 (74.1%) were detected in children aged 0-5 years, 51 (16.7%) in children aged 6-10 years, and 28 (9.2%) in children aged 11-14 years (Table 3). Co-infections between EPEC and either *Clostridioides difficile* (17 cases) or *Campylobacter* and NoV (16 cases each) prevailed in children aged 0-5 years (Table 3). Co-infections between EPEC and NoV (5 cases) predominated in children aged 6-10 years, while those between EPEC *and Campylobacter* (8 cases) in children aged 11-14 years.

3.3 Seasonality of enteric pathogens

The number of positive samples decreased from 53.9% (445/825) in 2018 and 56.1% (408/727) in 2019 to 48.2% (248/514) in 2020 (53.9% vs. 48.2%, $P=0.043$).

Supplementary table 1 shows the monthly distribution of all pathogens revealed, while Figure 2 that of the most common pathogens.

EPEC (Fig. 2A) was systematically detected except in March 2019, predominating in summer and early autumn. No seasonal trends were apparent for *Clostridioides difficile* (Fig. 2B), contrarily to *Campylobacter* and EAEC, showing increased rates during summer (Fig. 2D and 2H). *Salmonella* (Fig. 2G) increased its detection rate in summer of 2018 and 2020.

187 Seasonal prevalence was found for NoV in winter (Fig. 2C), while RV detection increased in spring
188 (Fig. 2E). AdV did not seem to vary hugely its percentage of positive cases during the study, but
189 two peaks passing the median more than twice were found in December 2018 (18%) and April 2019
190 (27%) (Fig. 2F).
191 Different trends were observed in 2020 during the SARS-CoV-2 lockdown (from March 9 to May
192 18) when compared to previous years. The circulation of AdV and EAEC (Fig. 2F and 2H) was
193 interrupted, while for EPEC, *Clostridioides difficile*, NoV, *Campylobacter*, and *Salmonella* (Fig.
194 2A, 2B, 2C, 2D, 2G) the detection rate decreased considerably under the median.

4. DISCUSSION

This study reveals the epidemiology of AGE pathogens in 2,066 children who underwent stool testing by FAGP from 2018 to 2020.

Molecular methods have greatly increased the sensitivity of pathogen detection expanding their involvement as AGE etiologic agents [14], although they do not yield an isolate and might detect nucleic acids from non-viable microorganisms unrelated to illness. Multiplex PCR assays may present limitation in finding the true etiologic agent in case of multiple pathogens detection and need to perform culture isolation and antibiotics sensitivity evaluation in case of bacterial identification [14].

FAGP has been usefully included in our diagnostic algorithm [10], with the goal of limiting the use of conventional methods in light of its high sensitivity and reproducibility [15].

In the study population, 53.3% of the samples resulted positive in agreement with previous data [10,15]. Of the positive samples, 68.2% were from children aged 0-5 years.

FAGP detected a higher frequency of single infections (72.3%) mostly caused by bacteria (59.2%), also consistent with literature data [16]. The more prevalent pathogens were EPEC (21.14%), *Clostridioides difficile* (11.5%), NoV (11.44%), and *Campylobacter* (10.17%).

Clostridioides difficile and *Campylobacter* showed higher rates than in our previous survey [10]. In contrast, RV decreased its frequency, as a likely consequence of the vaccine administration [14,17]. Antithetical considerations apply to NoV, being the vaccine candidates under study.

Although EPEC and EAEC were frequently detected, their etiological role remains unclear [18,19]. Interestingly, AGE-related inflammation modulates the intestinal microbiota, favoring the unusual growth of *Enterobacteriaceae* [20].

Clostridioides difficile was the second most common pathogen. This finding is difficult to interpret, given the high rate of asymptomatic carriage in young infants (<12 months) and frequent co-detection of other pathogens [21,22]. Therefore, testing should be performed when specifically requested and consultation with the pediatrician is recommended [22]. In the study population, only

13 samples had appropriate clinical information (data not shown). Being *Clostridioides difficile* more frequently detected in single infections, antibiotic treatments may have favored its proliferation, also considering the low rate (25%) of toxigenic samples.

Campylobacteriosis shows high incidence in the study population, contrarily to previous results [23]. In this regard, Italian data are likely to be underestimated, being not a statutory notification illness. Other Authors showed that *Campylobacteriosis* is increasing [24].

Important, FAGP allowed detecting *Plesiomonas shigelloides*, which is frequently unnoticed by conventional methods [25].

A frequent co-detection of viruses and bacteria was assessed especially in young children, according to Park et al. [26]. Although co-infecting viral and bacterial pathogens cause more severe gastroenteritis with higher probability of hospitalization [27], the knowledge on their etiological role remains incomplete [28]. Overall, *Escherichia coli* was the most prevalent pathogen in co-infections, according to Vergadi et al. [28].

EPEC, *Campylobacter* and EAEC predominated in summer, NoV in winter, and RV in spring. These seasonality differences confirm the need of continuous surveillance. These findings were in agreement with previous studies [10,29,30], but conflicting results were reported [31,32].

During the lockdown imposed because of the SARS-CoV-2 pandemic, care for children in Italy have been kept to minimal standards. Although gastrointestinal manifestations are observed during SARS-CoV-2 infection [33], in 2020 diminished both hospitalizations (342 in 2020 vs. 517 in 2018) and pathogens detection rates (22% in 2020 vs. 41.1% in 2018, $P<0.0001$). Individual protection measures and social distancing interrupted the circulation of AdV and EAEC, and decreased that of EPEC, *Clostridioides difficile*, NoV, *Campylobacter*, and *Salmonella*. Accordingly, lower rates of infectious disease transmission and delay in attendances to pediatric departments, with the tendency to seek hospital care only in case of severe or unremitting symptoms were described [34].

The reported findings focused on severe AGE cases, causing a high percentage of hospitalizations. The rapid diagnosis by FAGP helped the pediatricians in the management of patients, allowing a faster decision on the need for hospitalization. Since self-limited and asymptomatic cases were not examined, cautious epidemiological conclusions should be drawn.

Conclusions

Timely diagnosis improves the surveillance of the burden associated to AGE in children. Despite the costs of FAGP, the reduction in workload, antibiotic use and hospitalization renders this assay cost-effective. Our findings help to estimate the epidemiology of diarrhoea causative agents in children. Moreover, we tracked pathogen seasonality, reinforcing the need for accurate surveillance to control AGE.

REFERENCES

1. World Health Organization. Diharroeal disease 2017. https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease
2. Thielman NM, Guerrant RL. Clinical practice. Acute infectious diarrhea. The New England Journal of Medicine 2004;350:38–47. https://doi.org/10.1056/NEJMcp031534.
3. Das JK, Salam RA, Bhutta ZA. Global burden of childhood diarrhea and interventions. Current Opinion in Infectious Diseases 2014;27. https://doi.org/10.1097/QCO.0000000000000096.
4. Medici MC, Martinelli M, Abelli LA, Ruggeri FM, di Bartolo I, Arcangeletti MC, et al. Molecular epidemiology of norovirus infections in sporadic cases of viral gastroenteritis among children in Northern Italy. Journal of Medical Virology 2006;78. https://doi.org/10.1002/jmv.20723.
5. Medici MC, Martinelli M, Arcangeletti MC, Pinardi F, De Conto F, Dodi I, et al. Epidemiological aspects of human rotavirus infection in children hospitalized with acute gastroenteritis in an area of northern Italy. Acta Biomedica de l'Ateneo Parmense 2004;75.
6. Mattei A, Sbarbati M, Fiasca F, Angelone AM, Mazzei MC, di Orio F. Temporal trends in hospitalization for rotavirus gastroenteritis: A nationwide study in Italy, 2005–2012. Human Vaccines and Immunotherapeutics 2016;12. https://doi.org/10.1080/21645515.2015.1081726.
7. Elliott EJ. Acute gastroenteritis in children. British Medical Journal 2007;334. https://doi.org/10.1136/bmj.39036.406169.80.
8. Freeman K, Mistry H, Tsertsvadze A, Royle P, McCarthy N, Taylor-Phillips S, et al. Multiplex tests to identify gastrointestinal bacteria, viruses and parasites in people with suspected infectious gastroenteritis: A systematic review and economic analysis. Health Technology Assessment 2017;21. https://doi.org/10.3310/hta21230.

9. Calderaro A, Buttrini M, Martinelli M, Farina B, Moro T, Montecchini S, et al. Rapid classification of Clostridioides difficile strains using maldi-tof ms peak-based assay in comparison with PCR-ribotyping. Microorganisms 2021;9. https://doi.org/10.3390/microorganisms9030661.
10. Calderaro A, Martinelli M, Buttrini M, Montecchini S, Covan S, Rossi S, et al. Contribution of the FilmArray® Gastrointestinal Panel in the laboratory diagnosis of gastroenteritis in a cohort of children: a two-year prospective study. International Journal of Medical Microbiology 2018;308. https://doi.org/10.1016/j.ijmm.2018.04.007.
11. Calderaro A, Gorrini C, Montecchini S, Peruzzi S, Piccolo G, Rossi S, et al. Evaluation of a real-time polymerase chain reaction assay for the laboratory diagnosis of giardiasis. Diagnostic Microbiology and Infectious Disease 2010;66. https://doi.org/10.1016/j.diagmicrobio.2009.10.004.
12. Calderaro A, Montecchini S, Gorrini C, Dettori G, Chezzi C. Similar diagnostic performances of antigen detection and nucleic acid detection of Cryptosporidium spp. in a low-prevalence setting. Diagnostic Microbiology and Infectious Disease 2011;70. https://doi.org/10.1016/j.diagmicrobio.2010.11.017.
13. Calderaro A, Montecchini S, Rossi S, Gorrini C, De Conto F, Medici MC, et al. Intestinal parasitoses in a tertiary-care hospital located in a non-endemic setting during 2006-2010. BMC Infectious Diseases 2014;14. https://doi.org/10.1186/1471-2334-14-264.
14. Guarino A, Aguilar J, Berkley J, Broekaert I, Vazquez-Frias R, Holtz L, et al. Acute Gastroenteritis in Children of the World: What Needs to Be Done? Journal of Pediatric Gastroenterology and Nutrition 2020;70. https://doi.org/10.1097/MPG.0000000000002669.
15. Piralla A, Lunghi G, Ardissino G, Girello A, Premoli M, Bava E, et al. FilmArray™ GI panel performance for the diagnosis of acute gastroenteritis or hemorragic diarrhea. BMC Microbiology 2017;17. https://doi.org/10.1186/s12866-017-1018-2.

16. O'Neal M, Murray H, Dash S, Al-Hasan MN, Justo JA, Bookstaver PB. Evaluating appropriateness and diagnostic stewardship opportunities of multiplex polymerase chain reaction gastrointestinal testing within a hospital system. Therapeutic Advances in Infectious Disease 2020;7. https://doi.org/10.1177/2049936120959561.
17. Sabbatucci M, Odone A, Signorelli C, Siddu A, Maraglino F, Rezza G. Improved Temporal Trends of Vaccination Coverage Rates in Childhood after the Mandatory Vaccination Act, Italy 2014–2019. Journal of Clinical Medicine 2021;10. https://doi.org/10.3390/jcm10122540.
18. Beczkiewicz A, Cebelinski E, Decuir M, Lappi V, Wang X, Smith K, et al. High Relative Frequency of Enteroaggregative Escherichia coli among Patients with Reportable Enteric Pathogens, Minnesota, 2016-2017. Clinical Infectious Diseases 2019;69. https://doi.org/10.1093/cid/ciy890.
19. Hu J, Torres AG. Enteropathogenic Escherichia coli: Foe or innocent bystander? Clinical Microbiology and Infection 2015;21. https://doi.org/10.1016/j.cmi.2015.01.015.
20. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, et al. Host-Mediated Inflammation Disrupts the Intestinal Microbiota and Promotes the Overgrowth of Enterobacteriaceae. Cell Host and Microbe 2007;2. https://doi.org/10.1016/j.chom.2007.06.010.
21. Furuya-Kanamori L, Marquess J, Yakob L, Riley T v., Paterson DL, Foster NF, et al. Asymptomatic Clostridium difficile colonization: Epidemiology and clinical implications. BMC Infectious Diseases 2015;15. https://doi.org/10.1186/s12879-015-1258-4.
22. Sammons JS, Toltzis P. Pitfalls in Diagnosis of Pediatric Clostridium difficile Infection. Infectious Disease Clinics of North America 2015;29. https://doi.org/10.1016/j.idc.2015.05.010.

23. Lucarelli C, Dionisi AM, Trezzi L, Farina C, Passera M, Kärki T, et al. Molecular and epidemiological analysis of a campylobacter jejuni outbreak in Northern Italy in November 2013. Foodborne Pathogens and Disease 2016;13. https://doi.org/10.1089/fpd.2015.2104.

24. Platts-Mills JA, Kosek M. Update on the burden of Campylobacter in developing countries. Current Opinion in Infectious Diseases 2014;27. https://doi.org/10.1097/QCO.0000000000000091.

25. Michael Janda J, Abbott SL, McIver CJ. Plesiomonas shigelloides revisited. Clinical Microbiology Reviews 2016;29. https://doi.org/10.1128/CMR.00103-15.

26. Park S, Hitchcock MM, Gomez CA, Banaei N. Is follow-up testing with the filmarray gastrointestinal multiplex PCR panel necessary? Journal of Clinical Microbiology 2017;55. https://doi.org/10.1128/JCM.02354-16.

27. Valentini D, Vittucci AC, Grandin A, Tozzi AE, Russo C, Onori M, et al. Coinfection in acute gastroenteritis predicts a more severe clinical course in children. European Journal of Clinical Microbiology and Infectious Diseases 2013;32. https://doi.org/10.1007/s10096-013-1825-9.

28. Vergadi E, Maraki S, Dardamani E, Ladomenou F, Galanakis E. Polymicrobial gastroenteritis in children. Acta Paediatrica, International Journal of Paediatrics 2021;110. https://doi.org/10.1111/apa.15854.

29. Festini F, Cocchi P, Mambretti D, Tagliabue B, Carotti M, Ciofi D, et al. Nosocomial Rotavirus Gastroenteritis in pediatric patients: A multi-center prospective cohort study. BMC Infectious Diseases 2010;10. https://doi.org/10.1186/1471-2334-10-235.

30. Medici MC, Tummolo F, Martella V, De Conto F, Arcangeletti MC, Pinardi F, et al. Emergence of novel recombinant GII.P16_GII.2 and GII.P16_GII.4 Sydney 2012 norovirus strains in Italy, winter 2016/2017. New Microbiologica 2018;25. https://doi.org/10.1007/s12560-017-9278-4.

31. Amisano G, Fornasero S, Migliaretti G, Caramello S, Tarasco V, Savino F. Diarrheagenic escherichia coli in acute gastroenteritis in infants in north-west Italy. New Microbiologica 2011;34.
32. Iannetti S, Calistri P, Di Serafino G, Marotta F, Alessiani A, Antoci S, et al. Campylobacter jejuni and Campylobacter coli: prevalence, contamination levels, genetic diversity and antibiotic resistance in Italy. Veterinaria Italiana 2020;56. https://doi.org/10.12834/VetIt.1819.9596.
33. Matthai J, Shanmugam N, Sobhan P. Coronavirus Disease (COVID-19) and the Gastrointestinal System in Children. Indian Pediatrics 2020;57. https://doi.org/10.1007/s13312-020-1851-5.
34. Rusconi F, Di Fabrizio V, Puglia M, Sica M, De Santis R, Masi S, et al. Delayed presentation of children to the emergency department during the first wave of COVID-19 pandemic in Italy: Area-based cohort study. Acta Paediatrica 2021;110. https://doi.org/10.1111/apa.16019.

FIGURES AND TABLES LEGENDS

Figure 1. Distribution of enteric pathogens revealed by FAGP in stool samples from 2,066 children. The number of pathogens detected per year is shown below the histograms. The prevalence of each pathogen (%) was evaluated in reference to the total number of pathogens detected in the study period.

Figure 2. Monthly distribution of the most frequently detected pathogens in stool samples from 2,066 children. A: EPEC; B: *Clostridioides difficile*; C: Norovirus; D: *Campylobacter*; E: Rotavirus; F: Adenovirus; G: *Salmonella*; H: EAEC.

Table 1. Demographic data of the 2,066 children with AGE whose stool samples were analyzed with FAGP from 2018 to 2020.

Table 2. Different combinations of 305 co-infections detected in 1,101 positive stool samples.

Table 3. Most frequent co-infections detected in 2,066 children divided by age.

Author's contributions

Flora De Conto: Conceptualization, Methodology, Data acquisition, Writing, Editing, Validation, and Resource; Sharon Di Stefano: Investigation, Data curation and Visualization; Mirko Buttrini: Data curation, Methodology, and Visualization; Clara Maccari: Data curation; Maria Cristina Arcangeletti: Data acquisition and curation; Adriana Calderaro: Data acquisition, Reviewing, and Validation; Carlo Chezzi: Reviewing and Validation.

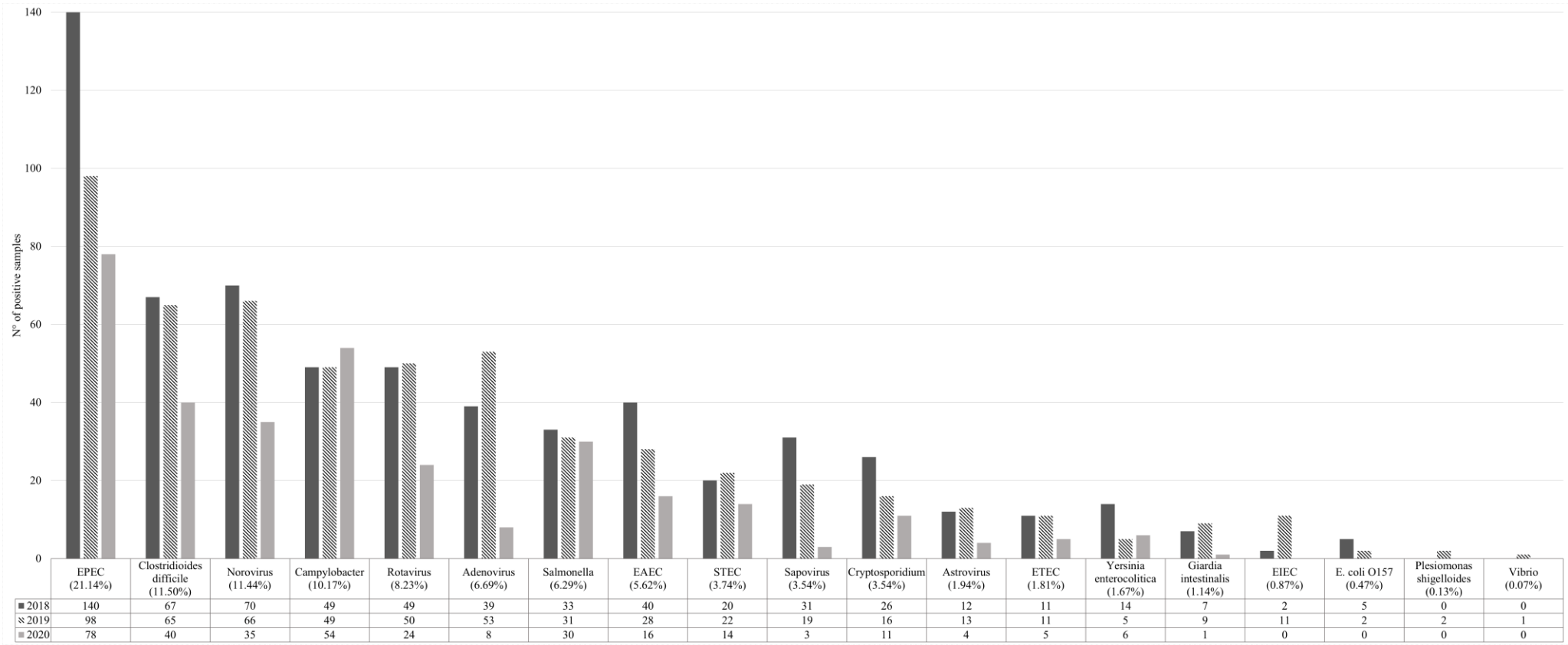
Ethical approval

This article does not describe any studies with human participants or animals.

The samples were sent to the University-Hospital of Parma for diagnostic purposes. Ethical approval at the University-Hospital of Parma is required when samples are used for applications other than laboratory diagnosis.

Figure 1

Click here to access/download;Figure;Figure 1.docx



	EPEC (21.14%)	Clostridioides difficile (11.50%)	Norovirus (11.44%)	Campylobacter (10.17%)	Rotavirus (8.23%)	Adenovirus (6.69%)	Salmonella (6.29%)	EAEC (5.62%)	STEC (3.74%)	Sapovirus (3.54%)	Cryptosporidium (3.54%)	Astrovirus (1.94%)	ETEC (1.81%)	Yersinia enterocolitica (1.67%)	Giardia intestinalis (1.14%)	EIEC (0.87%)	E. coli O157 (0.47%)	Plesiomonas shigelloides (0.13%)	Vibrio (0.07%)
2018	140	67	70	49	49	39	33	40	20	31	26	12	11	14	7	2	5	0	0
2019	98	65	66	49	50	53	31	28	22	19	16	13	11	5	9	11	2	2	1
2020	78	40	35	54	24	8	30	16	14	3	11	4	5	6	1	0	0	0	0

Figure 1

Figure 2

Click here to access/download;Figure;Figure 2.docx

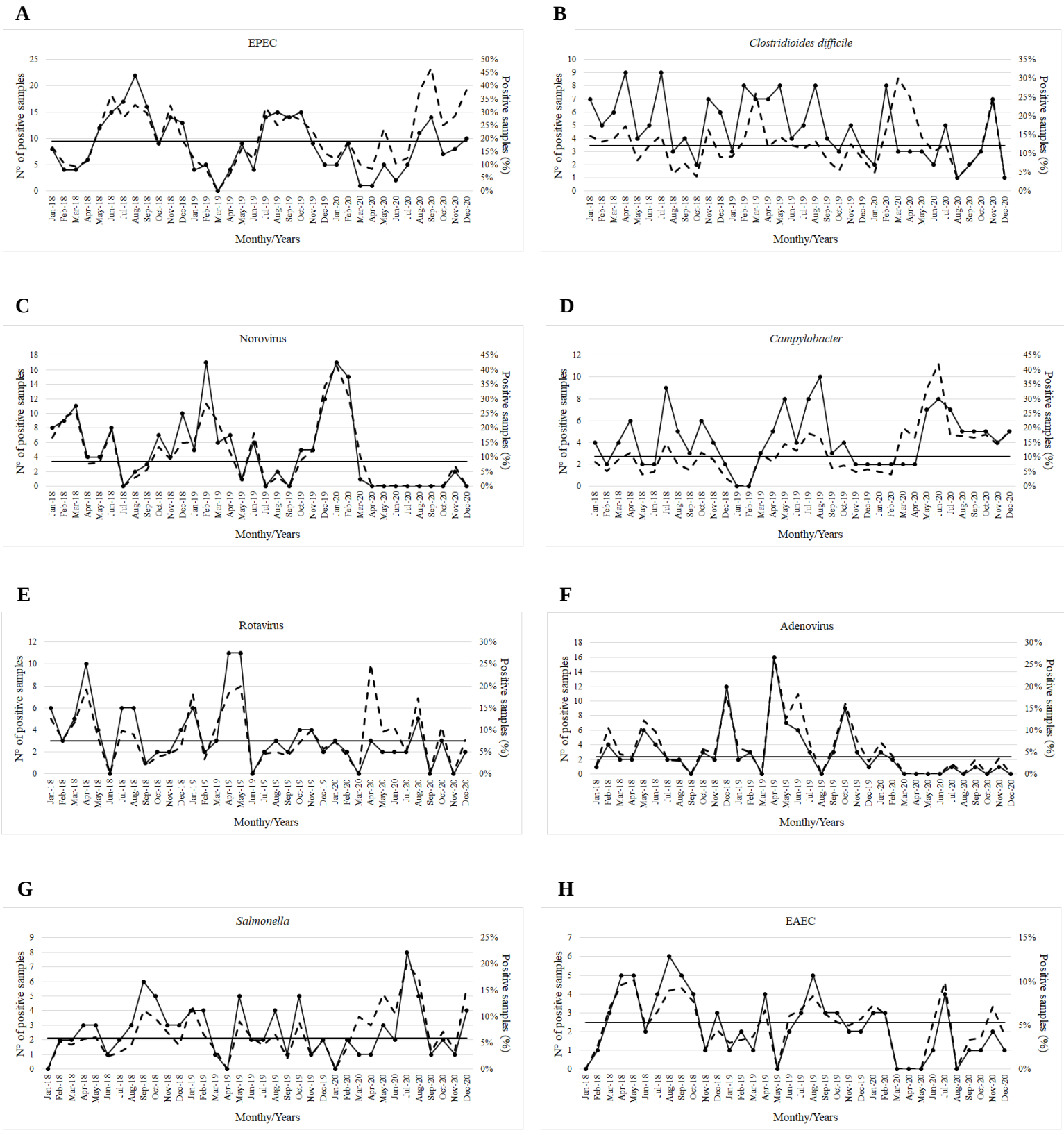


Figure 2

Table 1

Features	Number (%)
Males	1174 (56.8)
Females	892 (43.2)
Mean age (years) ± SD	4.4 ± 4.2
Median age (years)	2.9
0-5 years	1350 (65.3)
6-10 years	431 (20.9)
11-14 years	285 (13.8)
Inpatients	1295 (62.7)
Outpatients	771 (37.3)

SD - standard deviation

Table 2

Two pathogens	**N°**
Campylobacter + EPEC	24
Clostridioides difficile + EPEC	19
EPEC + NoV	16
EAEC + EPEC	11
Salmonella + STEC	11
AdV + EPEC	10
Cryptosporidium + EPEC	8
Clostridioides difficile + RV	8
AdV + NoV	7
AdV + RV	7
Campylobacter + *Clostridioides difficile*	7
Clostridioides difficile + NoV	6
Clostridioides difficile + SaV	6
EAEC + NoV	5
NoV + RV	5
EPEC + SaV	5
EPEC + *Yersinia enterocolitica*	5
Campylobacter + STEC	4
Less frequent infections with two pathogens	63
Total	81
Three pathogens	
Campylobacter + *Clostridioides difficile* + EPEC	4
AdV + *Clostridioides difficile* + EPEC	3
EAEC + ETEC + STEC	3
AdV + *Campylobacter* + EPEC	2
Campylobacter + EPEC + RV	2
Clostridioides difficile + EPEC + NoV	2
Cryptosporidium + ETEC + EPEC	2
EPEC + NoV + RV	2
Less frequent infections with three pathogens	46
Total	54
More than three pathogens	
AdV + EAEC + EPEC + RV	1
AdV + EPEC + ETEC + SaV	1
Campylobacter + EAEC + EIEC + *Salmonella*	1
Campylobacter + EAEC + EPEC + NoV	1
Campylobacter + EAEC + EPEC + RV	1
Clostridioides difficile + EAEC + EPEC + RV	1
Cryptosporidium + EAEC + EIEC + *Salmonella*	1
Cryptosporidium + EAEC + EPEC + SaV	1
EAEC + EPEC + ETEC + NoV	1
EAEC + EPEC + ETEC + NoV + SaV	1
EAEC + EPEC + NoV + RV	1
EAEC + STEC + EIEC + *Salmonella*	1
Total	12
Total	**147**

Table 3

Co-infections	N°	Age group (years)
EPEC		
Clostridioides difficile	17	
Campylobacter	16	
NoV	16	
AdV	10	
EAEC	8	
Clostridioides difficile		
RV	8	
AdV	7	
NoV	6	**0-5**
Campylobacter	6	
Campylobacter + EPEC	4	
NoV		
AdV	6	
Clostridioides difficile	6	
EPEC		
NoV	5	
EAEC	3	
Cryptosporidium	2	**6-10**
Clostridioides difficile	2	
EPEC		
Campylobacter	8	**11-14**
Total	**130**	

Supplementary table 1. Monthly distribution of pathogens detected by the FAGP in 2,066 children with AGE.

2018	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%
EPEC	8	4	4	6	12	15	17	22	16	9	14	13	**140**	**23**
Norovirus	8	9	11	4	4	8		2	3	7	4	10	**70**	**11.3**
Clostridioides difficile	7	5	6	9	4	5	9	3	4	2	7	6	**67**	**11**
Campylobacter	4	2	4	6	2	2	9	5	3	6	4	2	**49**	**8**
Rotavirus	6	3	5	10	4		6	6	1	2	2	4	**49**	**8**
Adenovirus	1	4	2	2	6	4	2	2		3	2	12	**40**	**6.5**
EAEC		1	3	5	5	2	4	6	5	4	1	3	**39**	**6.3**
Salmonella		2	2	3	3	1	2	3	6	5	3	3	**33**	**5.3**
Sapovirus	7	3	3	3	2				2	3	1	7	**31**	**5**
Cryptosporidium					1	2	5	8	3	3	3	1	**26**	**4.2**
STEC	3	1	1	1		2	2		4	6			**20**	**3.2**
Yersinia enterocolitica	1			2	3		2	3	1	1	1		**14**	**2.2**
Astrovirus	3	2	1	1	3							2	**12**	**2**
ETEC			1				1	3	3			3	**11**	**1.8**
Giardia intestinalis		1					1	1	2	1		1	**7**	**1.1**
E. coli O157		1					1	2	1				**5**	**0.8**
EIEC								1			1		**2**	**0.3**
Total													**615**	

2019	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%
EPEC	4	5		4	9	4	14	15	14	15	9	5	**98**	**18**
Norovirus	5	17	6	7	1	6		2		5	5	12	**66**	**12**
Clostridioides difficile	3	8	7	7	8	4	5	8	4	3	5	3	**65**	**11.8**
Adenovirus	2	3		16	7	6	3		3	9	3	1	**53**	**9.6**
Rotavirus	6	2	3	11	11		2	3	2	4	4	2	**50**	**9**
Campylobacter			3	5	8	4	8	10	3	4	2	2	**49**	**8.8**
Salmonella	4	4	1		5	2	2	4	1	5	1	2	**31**	**5.6**
EAEC	1	2	1	4		2	3	5	3	3	2	2	**28**	**5**
STEC		2				1	3	4	6	3	3		**22**	**4**
Sapovirus	4	8	1	1	2				1			2	**19**	**3.4**
Cryptosporidium					1	1	2	4	3	3	1	1	**16**	**3**
Astrovirus	1	3		3	2	1					1	2	**13**	**2.3**
EIEC	1	3		2				1	2	1		1	**11**	**2**
ETEC	1		1			1		3	2	1	2		**11**	**2**
Giardia intestinalis		2	3				1	1			2		**9**	**1.6**
Yersinia enterocolitica		1			1	1	1		1				**5**	**1**
E. coli O157									2				**2**	**0.4**
P. shigelloides	1		1										**2**	**0.4**
Vibrio									1				**1**	**0.1**
Total													**551**	

2020	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%
EPEC	5	9	1	1	5	2	5	11	14	7	8	10	**78**	**23.7**
Campylobacter	2	2	2	2	7	8	7	5	5	5	4	5	**54**	**16.4**
Clostridioides difficile	2	8	3	3	3	2	5	1	2	3	7	1	**40**	**12.1**
Norovirus	17	15	1								2		**35**	**10.6**
Salmonella		2	1	1	3	2	8	5	1	2	1	4	**30**	**9.1**
Rotavirus	3	2		3	2	2	2	5		3		2	**24**	**7.2**
EAEC	3	3				1	4		1	1	2	1	**16**	**5**
STEC		1			1	1	4		2	3		2	**14**	**4.2**
Cryptosporidium						1	3	1	3	3			**11**	**3.3**
Adenovirus	3	2					1		1		1		**8**	**2.4**
Yersinia enterocolitica	1		1	2			1					1	**6**	**2**
ETEC	2	1							1	1			**5**	**1.5**
Astrovirus	2	1	1										**4**	**1.2**
Sapovirus	1	2											**3**	**1**
Giardia intestinalis								1					**1**	**0.3**
Total													**329**	