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PATHOGENIC VIRUS AND BACTERIA ASSOCIATED WITH DIARRHEA AMONG CHILDREN IN HILLA PROVINCE, IRAQ

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ABSTRACT : In this study, viruses were identified on 88(58.66%) specimens were no growth on culture media by using cer test Rotavirus- Adenovirus Card test, the results revealed that 45(51.13%) specimen were related to virus infection, the sera of patients were showed anti-IgM to Rotavirus and adenovirus antibodies. The results showed that, out of 45 positive viruses, the IFA was detected 33(73.3%) positive for the Rotavirus and 12(26.6%) positive for Adenovirus. The automated VITEK 2 device using GP and GN-ID cards containing (64) biochemical tests were used to validate the isolates of bacteria from GTI. The findings indicate that 62 (41.33%) isolates have been confirmed with an excellent ID message confidence level (94 to 99.8% likelihood percentage). This method is characterized by quick bacterial detection. The results showed that 30(48.38%) *Escherichia coli* isolates followed by 9(14.52%) *Proteus mirabilis* isolates, 6(9.67%) for each *Salmonella typhimurium* isolates and *Staphylococcus aureus* isolates, 3(4.84%) for each *Aeromonas hydrophila* and *Enterobactor earogenes* isolates, 2 (3.23%) for each *Pseudomonas aeruginosa* and *Compylobater jejuni* isolates and one isolate (1.62%) for *Vibrio cholera*. DNA was extracted from all suspected isolates that previously identified as *E. coli* by the vitek2 system, DNA samples were used for PCR amplification of *eae* for EPEC and *stx1* for EHEC primers. Out of the 30 samples of *E. coli*, 18(60%) EPEC by the specific produced 482 bp DNA fragment, when compared with allelic ladder, and 12(40%) EHEC by the specific produced 348 bp.

Key words : Virus, indirect immunofluorescence antibody, diarrhea, PCR, bacteria.

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INTRODUCTION

In children below 5 years, diarrhea is one of the leading causes of death and poses a great challenge for children (Khalil *et al*, 2018). Globally, in children who are lower than three years of age acute infectious enteritisis is one of the common childhood diseases (Mokomane *et al*, 2018). This condition is characterized by frequent liquid or pasty stools, for more than thrice in 24 hours. This condition can be associated with or without fever or vomiting (Iftikhar *et al*, 2019). In usual cases, diarrhea normally lasts less than 7 days. It is referred to as protracted diarrhea if it lasts more than 14 days (Sumathi *et al*, 2018). Watery diarrhea, stomach cramps, nausea or vomiting, and occasionally fever are all symptoms of viral gastroenteritis (Demers-Mathieu *et al*, 2018). Even in infancy, gastrointestinal problems can arise frequently (Rossouw *et al*, 2021). A viral infection in the stomach is normally the cause (Bányai *et al*, 2018). Infections with rotavirus and Norwalk virus are common causes of gastroenteritis in infants and children (Pérez-Ortín *et al*, 2019). The most common way of developing viral gastroenteritis is either by coming in contact with an infected person or by ingestion of water or food contaminated with the virus (Yekta *et al*, 2020). In children, especially those who are lower than 5 years of age from a low economic background and suffering from malnutrition this condition is more common (Galgamuwa *et al*, 2017). The contamination can be traveled either through the fecal-oral route or through the contaminated food and water or indirectly through some vectors (Elsamadony *et al*, 2021). In rural areas chances of getting infected by contaminated food and water are high. Moreover, the pathogen gets directly or indirectly transmitted through the flies also (Siddiqui *et al*, 2020; Nguyen *et al*, 2021). Globally, the pathogen varies from region to region and the type of diarrhea can be severe in cases where there are confections rather than mono infections (Owada, 2019).

Aim of the study

The object of this learning is the detection of viruses and bacteria in children by immunofluorescence antibody technique, Vitek2 System and PCR technique.

MATERIALS AND METHODS

Patients and clinical specimens

Total 150 children with ages ranging from 1 week to 5 years were included in this study. Children who visited the clinic in Hilla city with diarrhea, for the period between November 2020 to March 2021 were included in this study.

Ethical approval

Prior to their inclusion in the report, valid consent was obtained from the children's parents. Each child was notified of the procedure before samples were obtained, ensuring that they understand the procedure to be performed. Parents were aware that they had the legal right to refuse to enroll their children in the study without facing any repercussions.

Blood samples

Venipuncture was performed for the collection of blood samples from the children (5ml). Then the sample was inserted slowly into the disposable tubes and allowed to clot at room temperature for 30 minutes. Centrifugation was done at 3000×g for approximately three minutes. After centrifugation, the sera were separated and stored at -20^{0}C for further analysis.

Stool swabs

Fecal samples were collected by 2 to 3 swabs, and placed in a container with transport medium (Cary–Blair), and taken to the laboratory. All culture media used in the study were prepared according to the procedures recommended by the manufacturing companies.

Identification of viruses

Viruses were identified using cer test Rotavirus-Adenovirus Card test, approximately 100 mg of stool specimen was transferred by a stick into the stool collection tube along with diluents. The tube was shacked in order to ensure proper sample dispersion. After that 4 drops of the solution provided with the kit were added into the circular window marked with an arrow, finally, the results were read at 10 minutes by observing the coloring bands as per the method used earlier (Zavala Gomez, 2017).

Detection of viruses by indirect immunofluorescence antibody technique

An indirect immunofluorescent assay (IFA) Kit was used for the simultaneous diagnosis in human serum of IgM antibodies. This reaction is based on the interaction of antibodies with antigen absorbed on the slide surfaces. In the washing step unbound immunoglobulins are removed. Interaction of antigen-antibody complexes with fluoresceinlabeled anti-human globulin gives the final fluorescence under microscope.

Identification of bacteria

Morphology and examination under microscopic

Based on its morphological properties, a single colony from each primary positive culture on blood, MacConkey, and nutrient agar is graded and examined by light microscope after being stained with Gram's stain. Biochemical tests were done and the identification was completed (McFadden, 2000; Collee *et al*, 2006). Identification of bacterial isolates we used the vitek2 method.

Identification of bacterial isolates with Vitek2 System

The samples were collected as follows according to production instructions in Vitek 2 clinical microbiology used as an automatic identification (ID) instrument device. For removal of pure colonies a sterile plastic stick applier was used.

Both isolates added to the machine prior to processing and inoculated cards were processed within 30 minutes of inoculation within the instrument. Bacterial suspensions using a vacuum chamber in the system is filled (inoculated) with GN and GP cards. The identification card was then placed next to the transfer tube, which was then inserted into the corresponding suspension tube, and the sample-containing check tubes were placed in a cassette.

The cassette could only accommodate 10 tubes. The filled cassette was then placed inside the vacuum chamber station. After application of vacuum, air was recharged into the station. This pushed the bacterial suspension into microchannels. Until loading inoculated cards into the circular incubator, a mechanism stops the transfer tube and seals the card. All card varieties were incubated at 35.5°C. Every 15 minutes, each card was removed from the incubator and transported to the reaction readings optical system, where it was returned to the incubator before the next read time.

DNA extraction from bacterial culture and specific primers

From the *E. coli* (EPEC, EHEC] isolates the genomic DNA was isolated as per the manufacturer protocol (Geneaid, USA). The details of the study (Galarce *et al*, 2019) are listed in Table 1.

Table 1 : The primers, sequences and PCR conditions.

Gene name	Primer sequence (5´- 3´)	Size of Bp	Conditions	References
eae* for *EPEC	F:TCAATGCAGTTCCGTTATCAGTT R:GTAAAGTCCGTTACCCCAACCTG	**482**	95ºC, 2 min. 95ºC, 30 sec. 62.7ºC decrease 0.5ºC per cycle, 30 sec.72ºC, 50.0 sec. Repeat steps 2-4 14 more times 95ºC, 30 sec. 55.7ºC, 30 sec. 72ºC, 50.0 sec. Repeat steps 6-8 19 more times 72ºC, 5 min. 4ºC, forever	Galarce *et al* (2019)
stx*$_1$ for *EHEC	F:CAGTTAATGTGGTGGCGAAGG R:CACCAGACAATGTAACCGCTG	**348**	95ºC, 2 min. 95ºC, 30 sec. 63.6ºC decrease 0.5ºC per cycle, 30 sec.72ºC, 40.0 sec. Repeat steps 2-4 14 more times 95ºC, 30 sec. 56.6ºC, 30 sec. 72ºC, 40.0 sec. Repeat steps 6-8 19 more times 72ºC, 5 min. 4ºC, forever	Galarce *et al* (2019)

RESULTS AND DISCUSSION

Identification of viruses by using cer test Rotavirus-Adenovirus card test and immunofluorescence antibody technique

In this study, in Table 2, viruses were identified on 88 (58.66%) specimens were no growth on culture media by using cer test Rotavirus- Adenovirus Card test, the results revealed that 45(51.13%) specimen were related to virus infection, the sera of patients were showed anti-IgM to Rota virus and Adenovirus antibodies. The results showed that, out of 45 positive viruses, the IFA was detected 33 (73.3%) positive for Rotavirus and 12 (26.6%) positive for Adenovirus as shown in Table 3, Figs. 1, 2. The results were in disagreement with results obtain by Badry *et al* (2016) in Kurdistan Region, Iraq; who found that 13.21% of viruses were isolated from children suffering from diarrhea. AL-Sadawi *et al* (2017) found that viral infections were mainly identified in infants in Al-Najaf Province, Iraq (12%).

In 72% of the cases, adenovirus antigen was detected in the stool samples (Bányai *et al*, 2018). On the other hand, in 77% of the cases, antibody enzyme immunoassay was capable of detecting the IgM (AL-Sadawi *et al*, 2017). The IgM antibody response was inconsistent, with no direct link to the children's age or the seriousness of their clinical symptoms. IgM antibody responses were seen in 48 percent of the children, with IgM antibodies lasting approximately two months (Huang *et al*, 2020). All the pathogens present in the GIT, 5%-8% of the children, who are lower than 2 years of age contains Adenoviruses (Carcillo *et al*, 2017). Detection of HAdV infections is difficult to detect as they have similar clinical symptoms as other GTT viruses (Marchio *et al*, 2018).

Hence, diagnostic approaches must be sensitive enough to detect the prolonged shedding of adenovirus in clinical specimens (Lee *et al*, 2020). Several methods are available for the detection of adenovirus infections, however, molecular methods are the most sensitive ones (Etemadi *et al*, 2019). In all children in both developing and developed countries, rotavirus infections are common in children aged 3 to 5 years (Sadiq *et al*, 2018). Neonatal infections do occur, but they are mostly asymptomatic or mild, likely due to maternal antibody protection. Clinical illness is most common in children aged 4–23 months, who are also at the highest risk of developing a serious illness that necessitates hospitalization (Zhang *et al*, 2020).

Several methods, including electron microscopy, polyacrylamide gel electrophoresis, antigen detection assays, reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation, can be used to detect rotavirus in stool specimens from children with gastroenteritis (Lorestani *et al*, 2019). Visualization of antigens using antibodies as fluorescent probes can be done Immunofluorescence assays (Schreiber and Smith, 2021). Immunofluorescence has proved to be effective in the determination of the cellular distribution of antigens even from frozen samples or in the identification of specific DNA sequences in a chromosome (Abu Abed and Brinkmann, 2019). The system has combined high

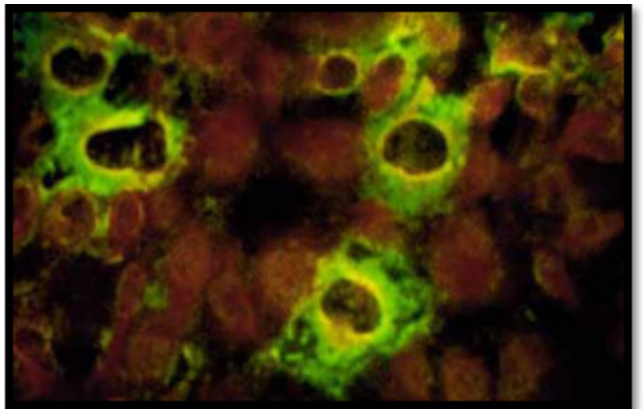

Fig. 1 : Detection of Rotavirus by immunofluorescent assay (magnification power 400x).

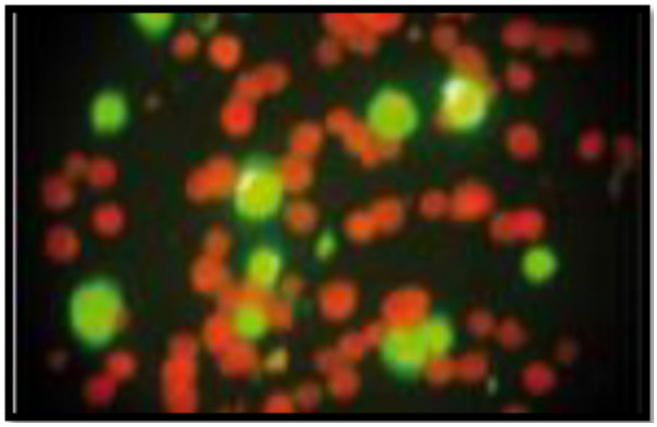

Fig. 2 : Detection of Adenovirus by immunofluorescent assay (magnification power 400x).

sensitivity with high resolution in the visualization of antigens, and it will be a significant tool for pathologists for several years. Immunofluorescence labeling of formalin-fixed, paraffin-embedded tissue or microbial antigens is the subject of a methodology paper (Parra *et al*, 2019).

Identification of pathogenic bacteria by Vitek2 System

The automated VITEK 2 device using GP, GN-ID cards containing (64) biochemical tests were used to validate the isolates of bacteria from GTI. The findings indicate that 62(41.33%) isolates have been confirmed with an excellent ID message confidence level (94 to 99.8% likelihood percentage). This method is characterized by quick bacterial detection. The results of the study were in agreement with results obtained by Al-Abbas (2018) in Karbala city, who showed that 37.65% were positive to bacterial growth. In Babylon Province, 60% of incidences were reported for bacterial infected diarrhea (Yasir, 2017).

Lanata *et al* (2013) have reported that another cause of diarrhea is increased use of antibiotics. In most of the viral and parasitic diarrhea in children under 5 years of

Table 2 : Viruses and bacteria isolates from stool samples.

No. of samples isolates	Viruses isolates	No. of bacteria
150	88 (58.66%)	62 (41.33%)
Total	150(100%)	

Table 3 : Viruses were isolated according to cer test Rotavirus-Adenovirus Card test.

No. of viruses	Rotavirus	Adenovirus
45 out of 88 samples with no growth on culture media	33(73.3%)	12(26.6%)
Total	**45(100%)**	

age mixed infections are also seen. In this study, the results showed that 30(48.38%) *Escherichia coli* isolates followed by 9(14.52%) *Proteus mirabilis* isolates, 6(9.67%) for each *Salmonella typhimurium* isolates and *Staphylococcus aureus* isolates, 3(4.84%) for each *Aeromonas hydrophila* and *Enterobactor earogenes* isolates, 2(3.23%) for each *Pseudomonas aeruginosa* and *Compylobater jejuni* isolates and one isolate (1.62%) for *Vibrio cholera*. The results were shown in Table 4. These results were in agreement with results obtained by Badry *et al* (2014) in Kurdistan Region-Iraq who found that the most prevalent enteric pathogens were *Escherichia coli* in 58.43%. Hundreds of harmless *E. coli* strains are present in the healthy human intestines. However, only one toxic strain can cause severe stomach upset (Al-Abbas, 2018). Mobley *et al* (2019) found that *Proteus* species have been associated with infectious gastroenteritis. *P. mirabilis* was more prevalent in children with diarrhea at 10.8%.

Proteus mirabilis is an opportunistic pathogen that causes GTI in humans. Some strains of *P. mirabilis* were also shown to be associated with food poisoning (Lanata *et al*, 2013). Another bacteria called *Salmonella* causes salmonellosis. Maximum of the children develop symptoms of diarrhea 12 to 72 hours after getting infected with these bacteria (Mobley, 2019). In healthy humans also this bacteria is present in the intestinal tracts, after ingestion of food contaminated with the animal feces the infection happens. In most cases foods like poultry, beef, eggs and milk get contaminated (Chlebicz and Œli¿ewska, 2018). Another type of bacterial infection, Campylobacteriosis causes diarrhea, stomach cramps, and fever. Children remain contagious for about two to five days when they have all these symptoms (Same and Tamma, 2018). The infection is transmitted by consuming meat infected with *Campylobacter jejuni* bacteria or by drinking contaminated water. Other sources of contamination include raw eggs and unpasteurized milk (Alegbeleye *et al*, 2018). In children, a wide spectrum of

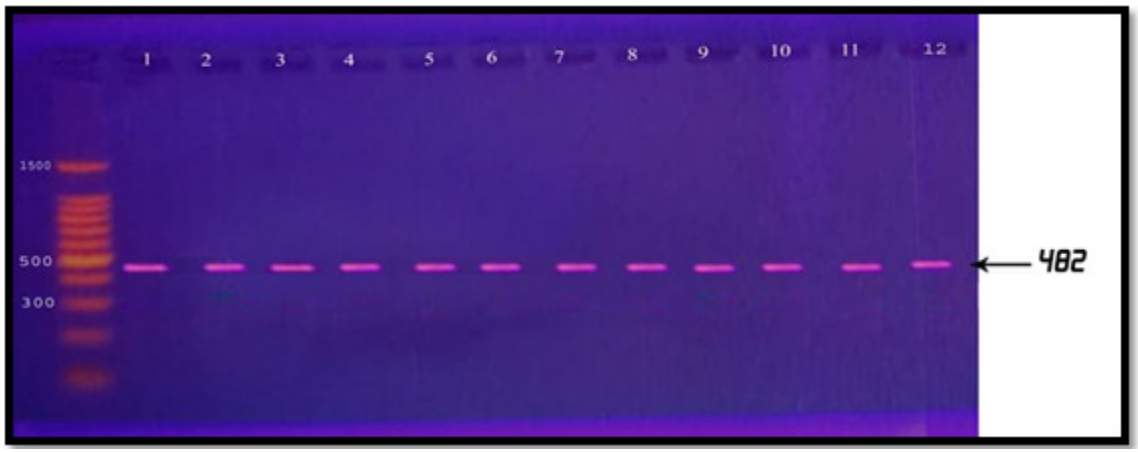

Fig. 3 : Agarose gel electrophoresis (1.5%) of PCR amplified of *eae* gene (482)bp of *EPEC* for (55)min at (70) volt. L: ladder (DNA marker), (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) Amplify of *eae* gene in clinical isolates of *EPEC*.

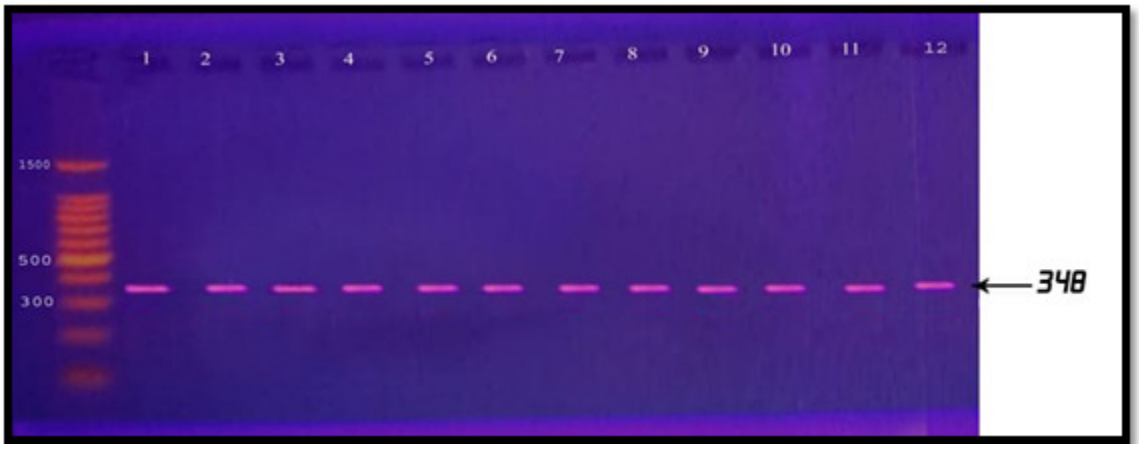

Fig. 4 : Agarose gel electrophoresis (1.5%) of PCR amplified of *stx1* gene (348)bp of *EHEC* for (55)min at (70) volt. L: ladder (DNA marker), (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) Amplify of *stx1* gene in clinical isolates of *EHEC*.

bacteria causes diarrhea including *Salmonella* spp., *E. coli*, *Yersiniaenterocolitica, Shigella* spp., *Proteus* spp., *Klebsiella* spp. and *Citrobacter* spp. (Ng'ang'a, 2018). *Vibrio cholera* causes severe watery diarrhea (Rodriguez and Kahwaji, 2020). During the first year of life, infection by EPEC and rotavirus are common and with age, the incidence of infection also decreases. While the incidence of infection by *Salmonella* spp. increases. In children *Shigella* spp. usually causes bloody diarrhea, whereas, *Salmonella* spp, Bacteria EPEC and *Campylobacter* spp. usually causes watery diarrhea (Mathew *et al*, 2019). Other bacteria usually cause mixed infections (Man *et al*, 2019). Children, who were only partially or not at all vaccinated increased their risk of infected diarrhea because the lack of vaccination rendered them more vulnerable to diseases such as whooping cough and measles. This would hurt one's wellbeing, making them vulnerable to diarrhea and other diseases (Di Pietrantonj *et al*, 2020).

Detection of *eae and stx1* genes by PCR

The polymerase chain reaction technique relies on DNA polymerase's ability to synthesize new strands of DNA that are complementary to the provided template strand, and after the PCR reaction, the same sequence will have been accumulated in billions of copies. Using the vitek2 system DNA was purified from all the suspected *E. coli* and *Aeromonas hydrophila* isolates by using conventional PCR, *eae* for EPEC and stx_1 for EHEC primers; according to the criteria mentioned in Table (1). Gen electrophoresis showed that only 60% of the samples as EPEC by the production of 482 bp DNA fragment when compared with allelic ladder, and 12(40%) EHEC by the specific produced 348 bp as shown in Figs. 3, 4.

These results were in agreement with results obtained by Chellapandi *et al* (2017), who found that in young children, EPEC is an important diarrheal pathogen. There has been a significant shift in EPEC prevalence as the diagnosis is now primarily based on molecular criteria. EPEC was one of the diarrheagenic *E. coli* pathotypes and it is one of the most common pathogens infecting children worldwide due to its high prevalence in the population and hospital environment, as well as the fact

Table 4 : Main pathogenic bacteria were detected from stool samples.

No. of samples	Type of bacteria	No. of bacterial isolates	%
62	*Escherichia coli*	30	48.38
	Proteus mirabilis	9	14.52
	Salmonella typhimurium	6	9.67
	Staphylococcus aureus	6	9.67
	Aeromonas hydrophila	3	4.84
	Enterobactorearogenes	3	4.84
	Pseudomonas aeruginosa	2	3.23
	Compylobaterjejuni	2	3.23
	Vibrio cholera	1	1.62
Total		**62**	**100%**

that it is one of the leading causes of chronic diarrhea (Zhou *et al*, 2018).

Carlino *et al* (2020) found that, in children with diarrhea, in 50.1% of cases the most common was EPEC, followed by EHEC (44%). Summer diarrhea in infants and neonatal diarrhea are also caused by EPEC. Because molecular diagnosis is now the primary method of diagnosing these pathogens, there has been a significant shift in the prevalence and distribution of these pathogens (Awad *et al*, 2020). The strain Enterohemorrhagic *E. coli* EHEC was the most common cause of illness in humans. It differed from other *E. coli* strains in that it releases Shiga toxin, a potent toxin. This toxin destroys the intestinal lining, resulting in bloody diarrhea (Kim *et al*, 2020). EHEC bind to the terminal ileal and colonic mucosa and releases toxins such as verocytotoxin (VT) 1 and 2 (Fan *et al*, 2019). These cause hemorrhagic colitis by killing colonic enterocytes. VT may also damage vascular endothelial cells, resulting in hemolytic uremic syndrome (Muhammad *et al*, 2017; Zaboon *et al*, 2021).

CONCLUSION

Viral infections are higher in children under the age of five years old than bacterial infections. Rotavirus and Adenovirus are the most important and common in children with diarrhea and *Escherichia coli* is the most common pathogen in these infections.

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