

# Viral and Bacterial Etiology of Acute Diarrhea among Children under 5 Years of Age in Wuhan, China

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## Abstract

**Background:** Acute diarrhea remains the serious problem in developing countries, especially among children under 5 years of age. Currently, only two or three common diarrhea pathogens were screened at most hospitals in China. The aim of this study was to provide a wide variety of diarrhea pathogens and their antimicrobial resistance patterns in children under 5 years of age.

**Methods:** Totally 381 stool samples collected from Tongji Hospital between July 1, 2014 and June 30, 2015 were tested by culture and/or polymerase chain reaction for eight kinds of bacteria and five kinds of viruses. An antimicrobial sensitivity test was performed using dilution method recommended by the Clinical and Laboratory Standards Institute.

**Results:** Viral infections were mainly identified in infants (0–11 months), whereas bacterial infections were more prevalent in the age of 24–59 months. About 69.8% of samples were positive for at least one pathogen, 51.7% of samples were virus positive, followed by bacteria positive cases (19.4%), and 12.6% of cases displayed co-infections with two viruses or a virus and a bacterium. Rotavirus was the most prevalent pathogen, followed closely by norovirus, while *Salmonella* was the most commonly isolated bacteria, followed by diarrheagenic *Escherichia coli* (DEC) and *Campylobacter*. More than 40% of *Salmonella* spp. and DEC isolates were resistant to first-line antibiotics (ampicillin, trimethoprim-sulfamethoxazole, and tetracycline). Around 10% of *Salmonella* spp. isolates were resistant to ceftriaxone and ciprofloxacin simultaneously. *Campylobacter* spp. displayed high resistance to ciprofloxacin but kept low resistance to azithromycin and doxycycline.

**Conclusions:** The etiology of acute diarrhea varies in children of different age groups. The high frequency of infection with viruses suggests the urgent demand for new viral vaccine development. Proper use of antibiotics in the treatment of acute diarrhea is crucial due to the high level of antibiotic resistance.

**Key words:** Acute Diarrhea; Bacteria; Children; Etiology; Virus

## INTRODUCTION

Diarrhea remains the serious problem globally due to its leading cause of death for children, especially among children under 5 years of age. Walker *et al.*<sup>[1]</sup> reported that there were 1.731 billion episodes of diarrhea leading to more than 700,000 deaths worldwide in 2011. In the past two decades, to reduce the burden of diarrhea, the Chinese government has made a great progress to improve public hygiene, which resulted in a remarkable decrease of mortality rate in children.<sup>[2]</sup> Despite these efforts, China is still 1 of 15 high-incidence countries (0.7 episodes per person-year).<sup>[1]</sup> Diarrhea ranked top 3 among the notifiable infectious disease by far.<sup>[3]</sup>

In China, diarrhea is the most common reason for children to visit health-care clinics, and the antibiotic resistance has

become a growing problem due to misuse of antibiotics. However, not all the bacterial or viral diarrhea pathogens causing diarrhea can be detected due to the technical limitation. In most clinical laboratories, only culture or immunological technique was used to screen for common diarrheal pathogens, such as *Salmonella*, *Shigella*, and/or rotavirus. Data from these assays alone cannot show the full

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spectrum of diarrheal pathogens. In this study, following a literature review and based on experts' opinions, we detected a panel of proven and/or plausible diarrheal pathogens to fully understand the main causes of acute diarrhea in children under 5 years of age in Wuhan, China.

## METHODS

### Sample collection

Totally, 381 children under 5 years of age, who visited the outpatient clinic of Tongji Hospital, Tongji Medical College of Huazhong University of Sciences and Technology (Wuhan, China) with acute diarrhea between July 1, 2014 and June 30, 2015, were enrolled in this general surveillance. Acute diarrhea was defined as the passing of liquid, loose, mucoid, or bloody stool for three or more times daily, with an episode lasting no longer than 14 days. The children's parents verbally agreed to participate in this study. All their fecal samples were collected in sterile containers and subjected to culture and/or polymerase chain reaction (PCR) analysis at the clinical laboratory of Tongji Hospital. For bacteriological testing, samples were inoculated within 2 h on arrival at the laboratory. For virological testing, the samples must be kept at  $-20^{\circ}\text{C}$  without preservatives prior to processing within a week. All residues were kept at  $-70^{\circ}\text{C}$  for further analysis.

### Isolation and identification of bacteria

On arrival at the laboratory, samples were plated onto selective and differential culture media. For isolation of *Salmonella*, *Shigella*, diarrheagenic *Escherichia coli* (DEC), *Vibrio*, *Yersinia*, *Aeromonas* and *Plesiomonas*, MacConkey agar (Mac), xylose lysine desoxycholate medium, sorbitol Mac agar, thiosulfate citrate bile salt, and Columbia blood agar (Oxoid, UK) were used. Sulfa enrichment broth (SBG) (Qingdao HopeBio-Technology, China) and phosphate-buffered saline were used as enrichment media to improve the isolation of *Salmonella* and *Yersinia*, respectively. Cefoperazone deoxycholate agar and Skirrow's medium were used to culture *Campylobacter* spp. in a microaerophilic environment at  $42^{\circ}\text{C}$ .

After incubation, suspected colonies were submitted to biochemical test using Vitek 2-compact automated system (bioMérieux, Marcy-l'Etoile, France). For DEC and *Campylobacter*, bacteria colonies were pooled for DNA extraction. The DNA products were subjected to PCR analysis using the primers as reported previously.<sup>[4-7]</sup>

Serotyping of *Salmonella* spp. and *Shigella* spp. was performed by slide agglutination tests using commercially available antisera (*Salmonella* Diagnostic Antisera Kit, Statens Serum Institute SSI Diagnostica, Copenhagen, Denmark; and anti-*Shigella* antiserum from Lanzhou Institute of Biological Products Co., Ltd., Lanzhou, China).

### Virus detection

The presence of viral pathogens including rotavirus (Groups A, B, and C), norovirus (GI and GII), sapovirus, astrovirus, and adenovirus were detected by molecular

biological techniques. Nucleic acids were extracted from fecal suspensions in 0.9% physiological saline using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The products were then subjected to one-step reverse transcription-PCR with type-specific primers as reported previously.<sup>[8-11]</sup> All PCR-positive products were re-confirmed with DNA-sequencing.

### Antimicrobial susceptibility tests

As recommended by the Clinical and Laboratory Standards Institute (CLSI), the minimum inhibitory concentration (MIC) of antibiotics was performed using standard agar dilution technique, except for *Campylobacter*, for which broth microdilution method was used. The antimicrobials were bought from China Food and Drug Research Institute, China. MIC results were interpreted according to CLSI M100, 2015 and CLSI M45, 2006 (for *Campylobacter*) recommendations. The reference strains, ATCC25922, ATCC 35218, and ATCC 29213 were used as controls.

Since CLSI did not recommend azithromycin breakpoints for *Campylobacter* or *Salmonella* spp. (except for *Salmonella typhi*), we used the breakpoints of CDC epidemiological cutoff values (ECOFFs)<sup>[12]</sup> for *Campylobacter* and interpreted the results for *Salmonella* spp. according to breakpoints for *S. typhi*, CLSI M100, 2015.

## RESULTS

### Characteristics of patients

Besides diarrhea, vomiting was the most common clinical manifestation, almost 32.3% of cases (123/381) presented with concomitant vomiting. Fever was the second most common clinical manifestation, with 27.5% (105/381) of children complaining of low- or mid-grade fever. Diarrhea lasted 1–4 days in two hundred and seventy children, and 5–10 days in another one hundred children, prior to the hospital visit. Only 11 children had 10–14 days of diarrhea [Figure 1].

### Prevalence of enteropathogens among different age groups and different seasons

The age distribution of 381 case patients in this study was as following: 64.6% were aged 0–11 months, 22.3% were 12–23 months, and 13.1% were 24–59 months [Figure 2a].

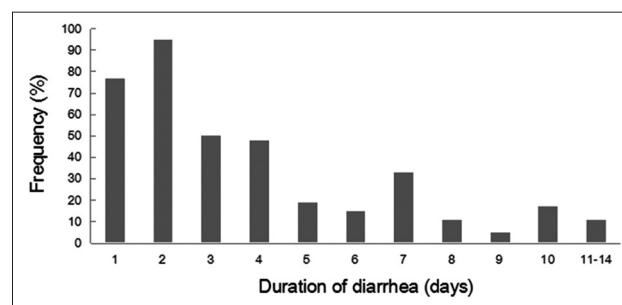


Figure 1: Duration of diarrhea in children under 5 years of age.

Viral agents (especially rotavirus and norovirus) were more likely to be identified in 0–11-month infants, while bacterial agents were more prevalent in the age group of 24–59 months [Figure 2b]. The seasonal distribution of the patients infected with virus or bacterium was also investigated. Viruses were prevalent in autumn months, while bacteria were prevalent in summer months [Figure 2c].

### Distribution of varied enteropathogens

As shown in Figure 3, at least one pathogen was tested positive in 266 (69.8%) samples from 381 children. Viruses took up the largest part of detectable enteropathogens with a positive rate of 51.7% (197/381), followed by bacteria with 19.4% of samples (74/381). There were 48 cases co-infected with two pathogens simultaneously. No specific pathogens were detected in 30.2% (115/381) of the samples [Figure 3a].

### Viral etiology

Rotavirus was the most commonly detected organism (40.8%, 98/240), followed by norovirus (36.7%, 88/240). These two viruses accounted for almost 80% of detectable viral diarrheal pathogens in this study. Among 98 rotavirus positive samples, Group A was the leading group identified, accounting for 65% (64/98), followed by Group B (22%, 22/98) and Group C (12%, 12/98). Among norovirus positive samples, GII was undoubtedly predominant, accounting for 95% (84/88) of these samples. Other viruses such as astrovirus, sapovirus, and adenovirus were detected less frequently (4% to 11%) [Figure 3b].

### Bacterial etiology

*Salmonella* was the most frequently identified organism, accounting for 43% of 74 bacterium-positive samples, followed by DEC (24%, 18/74). *Campylobacter* took the third place in bacteria inducing diarrhea, accounting for 15% (11/74), followed by *Aeromonas* (8%, 6/74), *Shigella* (7%, 5/74), *Vibrio* (1%, 1/74), and *Yersinia* (1%, 1/74). All *Campylobacter* identified in the current study were *Campylobacter jejuni* [Figure 3c].

### Co-infection

Forty-eight (12.6%, 48/381) cases displayed co-infection with two different enteropathogens. Co-infections with rotavirus and norovirus (44%, 21/48) were the most commonly identified, followed by co-infections of norovirus and adenovirus (19%, 9/48) and norovirus and sapovirus (15%, 7/48), successively [Figure 3d].

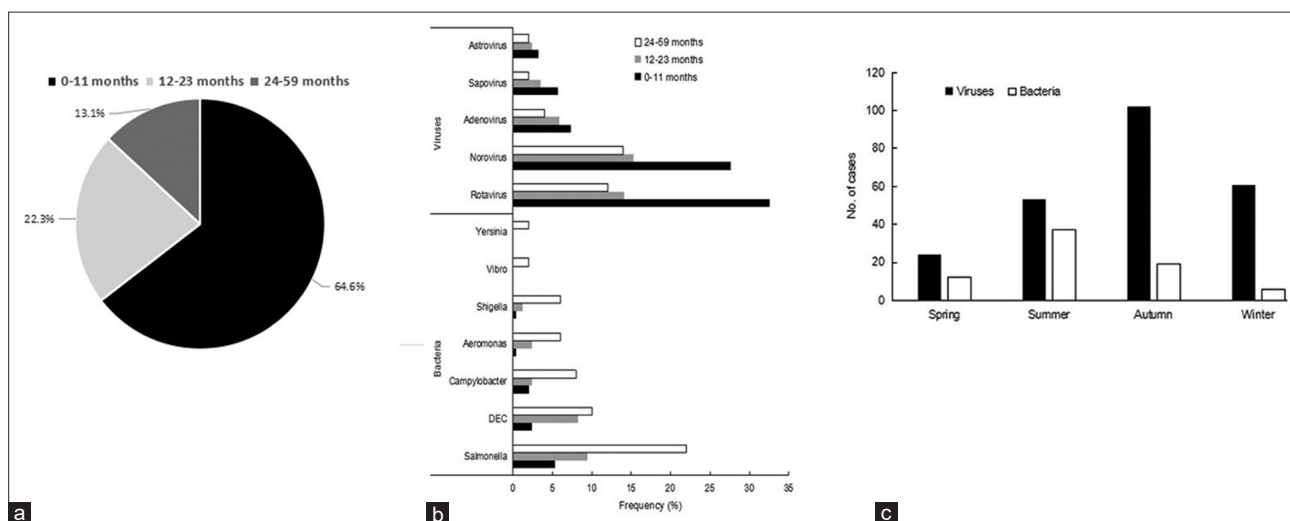
### Serotyping

Of the 32 *Salmonella* isolates, the predominant serotype was *Salmonella typhimurium* which accounted for more than 40% (13/32) of isolates, followed by *paratyphoid B* and *Salmonella enteritidis*. Both of them accounted for 13%. Of the 18 DEC isolates, enteropathogenic *Escherichia coli* (EPEC) and Shiga-toxin-producing *E. coli* (STEC) were the leading genotypes identified, account for 50% and 33%, respectively. Enteroggregative *E. coli* accounted for only 17%, while both enterotoxigenic *E. coli* and enteroinvasive *E. coli* were not identified. The predominant serotypes of *Shigella* isolates were *Shigella flexneri* (3/5) and *Shigella sonnei* (2/5). Neither *Vibrio cholera* nor O157:H7 was identified.

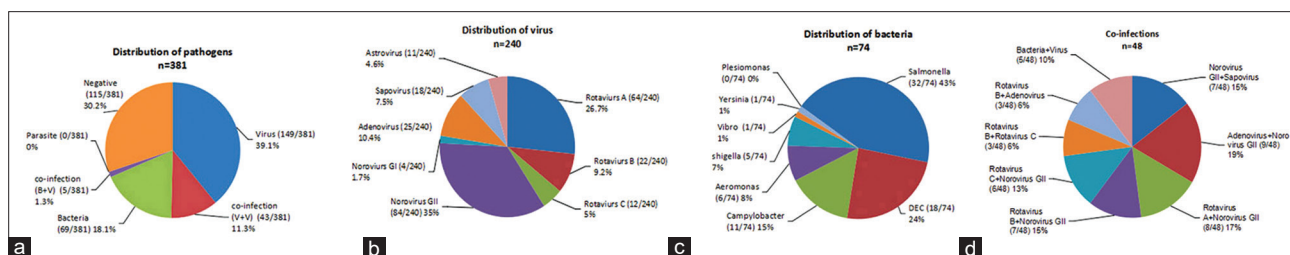
### Antimicrobial resistance

All bacteria were subjected to antimicrobial susceptibility test; however, only the data of *Salmonella*, DEC, and *Campylobacter* were shown because too few strains of other bacterial species were isolated.

As shown in Table 1, *Salmonella* exhibited a high frequency of resistance to the first-line antibiotics: ampicillin (68%) and trimethoprim-sulfamethoxazole (53%). Resistance rates to conventional and/or first-line antibiotics such as ciprofloxacin, levofloxacin, tetracycline, chloramphenicol, nalidixic acid, ceftriaxone, and azithromycin were 12%, 9%, 56%, 41%, 26%, 22%, and 16% respectively; 9% (3/32) of isolates were resistant to ceftriaxone and ciprofloxacin simultaneously.



**Figure 2:** Positive percentages of enteropathogens among children aged 0–59 months in acute diarrheal illnesses. (a) Patients with age distribution; (b) Prevalence of enteropathogens among different age groups; (c) Prevalence of enteropathogens among different seasons.



**Figure 3:** Distribution of diarrheal pathogens. (a) Distribution of pathogens; (b) Distribution of viruses; (c) Distribution of bacteria; (d) Co-infections.

**Table 1: Antimicrobial resistance rate of *Salmonella*, DEC, and *C. jejuni* (%)**

Antibiotics	<i>Salmonella</i> (n = 32)	DEC (n = 18)	<i>C. jejuni</i> (n = 11)
Ampicillin	68	67	–
Ampicillin/sulbactam	–	33	–
Nalidixic acid	26	–	–
Cefazolin	–	33	–
Cefuroxime	–	33	–
Cefotaxime	–	28	–
Ceftriaxone	22	–	–
Imipenem	–	0	–
Amikacin	–	17	–
Gentamicin	–	33	–
Ciprofloxacin	12	33	54
Levofloxacin	9	17	–
Chloramphenicol	41	33	–
Tetracycline	56	67	23
Trimethoprim-sulfamethoxazole	53	55	–
Azithromycin	16*	–	9†
Erythromycin	–	–	18
Doxycycline	–	–	0

–: Not tested. \*According to breakpoints for *Salmonella typhi*, CLSI M100, 2015. †According to breakpoints of Centers for Disease Control ECOFFs. ECOFFs: Epidemiological cutoff value; DEC: Diarrheagenic *Escherichia coli*; CLSI: Clinical and Laboratory Standards Institute; *C. jejuni*: *Campylobacter jejuni*.

Of the 18 DEC isolates, more than 50% were resistant to ampicillin, tetracycline, and trimethoprim-sulfamethoxazole. Approximately, 30% of isolates were resistant to ampicillin/sulbactam, cefazolin, cefuroxime, cefotaxime, gentamicin, ciprofloxacin, and chloramphenicol, and about 20% of isolates were resistant to amikacin and levofloxacin. All 18 isolates were sensitive to imipenem.

*Campylobacter* isolates (n = 11) showed low resistant to azithromycin, erythromycin, and doxycycline, with the rates of 9%, 18%, and 0, respectively; 54% of isolates were resistant to ciprofloxacin and 23% to tetracycline [Table 1].

## DISCUSSION

This study was conducted in central China to investigate a wide variety of diarrhea pathogens and their antimicrobial resistance patterns in children under 5 years of age. To get reliable data, both molecular and conventional techniques

were used to detect each sample. Furthermore, this study covered a whole year including the peak infection seasons, May to September.

Consistent with other studies,<sup>[13]</sup> our data showed that the most commonly detected pathogen causing diarrhea in children under 5 years of age were viruses. Among 381 samples, 197 (51.7%) were tested positive for at least one virus. Notably, infants (0–11 months) were more likely to be infected with viruses, especially rotavirus and norovirus, while bacterial agents were more prevalent in the age group of 24–59 months. This diversity of age distribution might reflect a natural change in host immunity and dietary. The seasonal distribution of viruses and bacteria was also different. Our data suggest that viruses were prevalent in autumn months, while bacteria were prevalent in summer months, which was consistent with previous studies.<sup>[14]</sup>

In this study, rotavirus was the most common diarrheal pathogen as reported by the previous studies.<sup>[13–16]</sup> However, a study from Africa showed bacteria were the prominent pathogens associated with diarrhea.<sup>[17]</sup> Norovirus took the second place in the list of most frequently detected diarrheal virus, which was similar to reports from other provinces of China.<sup>[15,18]</sup> Of 88 norovirus positive samples, 84 were identified as subtype GII, only 4 were subtype GI, which was similar to Anders *et al.*'s report.<sup>[19]</sup> Furthermore, of the 84 norovirus GII positive cases, 37 (44%) were co-infected with other diarrheal pathogens, while none of the norovirus GI cases was found to be co-infected with any other pathogens. The most frequently detected co-infections were rotavirus and norovirus, accounting for around 50% (44%, 21/48). Notably, the proportion of norovirus identified in this study was around 23.1% (88/381), which was much higher than the results of other studies (10%).<sup>[15,18]</sup> However, it was consistent with the report by Ramani *et al.*,<sup>[20]</sup> which showed that norovirus had replaced rotaviruses in recent years as the predominant viral pathogen in diarrheal children. The economic burden induced by norovirus infection inspired the urgent demand for norovirus vaccine in China. National Vaccine and Serum Institute has started research and development on norovirus vaccine.

In the present study, almost one-fifth of participants were infected with or carried one bacterial pathogen. It was noteworthy that the distribution of bacterial pathogens in different age groups was not even. Children aged



24–59 months were more likely to be infected with bacterial enteropathogens than other age groups. Among all identified bacterial enteropathogens in this study, *Salmonella* was the most frequently isolated bacterial pathogen which is usually prevalent in the developing countries such as India and Pakistan, while it is rare in industrialized countries.<sup>[21,22]</sup> A 5-year surveillance conducted in Shanghai, a highly developed area in China, demonstrated that the isolation rate of *Salmonella* decreased year by year,<sup>[23]</sup> which was attributed to the improvement of hygiene condition. Among the *Salmonella* positive samples in this study, the most frequently detected serotype was *S. typhimurium* which accounted for more than 40% of all *Salmonella* isolates. This was in agreement with previous reports from Niger and from Guangdong province in China. *S. typhimurium*, a multidrug-resistant strain, has been of concern to both veterinary and public health officials as an important swine and human pathogen. What is more, *S. typhimurium* infection has even become an increased risk to public health in some developed countries, such as UK and USA, accounting for nearly one-half of human *Salmonella* infections.<sup>[24]</sup> In our study, *Salmonella* exhibited a high frequency of resistance to the conventional first-line antibiotics: ampicillin, trimethoprim-sulfamethoxazole, and tetracycline (resistance rate  $\geq 50\%$ ). *Salmonella* also displayed increased resistance to third-generation cephalosporins, such as ceftriaxone (22%) and to fluoroquinolones, such as ciprofloxacin (13%) and levofloxacin (9%). Even worse is the growth of strains which were both resistant to third-generation cephalosporin and fluoroquinolones, accounting for around 10% (3/32). The rates of antibiotic resistance observed in our study were greater than that reported in a previous surveillance conducted in Shanghai, China.<sup>[25]</sup> Empirical antimicrobial therapy of severe *Salmonella* infection faces the challenge from the increasing resistance to extended-spectrum cephalosporins and ciprofloxacin.<sup>[26]</sup> However, 84% of *Salmonella* isolates were sensitive to azithromycin in this study, that is consistent with the previous studies<sup>[27,28]</sup> and suggests that azithromycin may be a good choice to treat bacterial gastroenteritis.

As shown in this study, DEC was the second most commonly isolated bacteria associated with children's diarrhea. EPEC was the most frequent isolated pathogen, which was consistent with other reports.<sup>[29,30]</sup> EPEC is usually prevalent in low-income countries where the poor hygiene condition contributes to its circulation. The prevalence of EPEC in central China suggests that the government needs to put further effort into improving the public health. STEC was the second frequently isolated pathogen in this study. Notably, STEC can lead to not only mild, self-limiting diarrhea but also hemorrhagic colitis and hemolytic uremic syndrome as a complication.<sup>[31]</sup> Its high isolation rate should be of concern to public health office. DEC also displayed high resistant to conventional and/or first-line-antibiotics (28–67%), low resistant to levofloxacin and amikacin (<20%), while all DEC strains were sensitive to imipenem. *Campylobacter* (2.8%, 11/381) was the third most frequently isolated bacteria causing diarrhea, even more than *Shigella* (1.0%, 4/381).

However, this number might be still underestimated in China as a result of the limited bacterial culture in the primary health-care facilities or use of antibiotics prior to visiting the hospital. In some developed countries, *Campylobacter* has become the leading bacteria causing acute diarrhea in children.<sup>[30,31]</sup> Our antimicrobial susceptibility tests showed that *Campylobacter* isolates displayed low-level resistance to azithromycin, erythromycin, and doxycycline (<10%). However, the ciprofloxacin resistance rate in *Campylobacter* isolates reached 54%, which is higher than that reported by Riley *et al.*<sup>[32]</sup> Moreover, the resistance rate of these isolates to tetracycline was 20%. Therefore, we should avoid using these two antibiotics to treat acute diarrhea.

As shown in the current study, viruses were the most predominant pathogenic agents causing acute diarrhea in children under 5 years of age in this region. Virus was more frequently isolated from infants than from the age group of 24–59 months, which was consistent with previous surveys from other cities of China.<sup>[15,18]</sup> However, the proportions of the two leading viral agents had changed. There was a considerable increase in the percentage of samples those were norovirus positive, compared to data from Chen *et al.*'s or Sai *et al.*'s studies.<sup>[15,18]</sup> This suggests that norovirus could replace rotavirus as the main cause of acute diarrhea in children under 5 years of age. Our study also indicated that a wide range of bacteria is responsible for acute diarrhea in children under 5 years of age. *Salmonella* and DEC continue to be the leading bacterial agents. Antimicrobial susceptibility screening indicated that ampicillin, trimethoprim-sulfamethoxazole, and tetracycline should not be used as the first-line therapeutic drugs for *Salmonella* spp. and DEC strains.

In summary, etiologic diagnosis of diarrhea is valuable for public health interventions. Our data can inform the development and implementation of diarrhea control and prevention programs in China. Antibiotics to treat bacterial diarrhea are widely available, but there are few vaccines for diarrhea-associated pathogens. This might explain the high incidence of acute diarrhea in children under 5 years of age, especially in rural areas, where sanitation is still lacking.<sup>[33]</sup> Until 2001, there was no rotavirus vaccine available in China.<sup>[2]</sup> The control of diarrhea cases heavily relied on the use of antibiotics. However, the high level of antibiotics resistance has become a serious problem in central China, challenging the effectiveness of antibiotic use.

This study had several limitations. It was a single-center study, and the number of cases was not as many as those reported in multicenter studies due to restrictions on time and resources. Nevertheless, this study will enrich the epidemiology database of China and will be of great importance for guiding the proper use of antibiotics.

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## Conflicts of interest

There are no conflicts of interest.

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