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Symptomatic and Asymptomatic Norovirus Infections among Hospitalized Young Children in Xi'an, China

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MD

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2005

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Abstract

Symptomatic and Asymptomatic Norovirus Infections among Hospitalized Young Children in Xi'an, China

By Changxin Dong

Background: Acute gastroenteritis is a major cause of childhood morbidity and mortality in the world. Noroviruses (NoVs) have been recognized as a common etiology of acute gastroenteritis in children, but NoVs genotyping and epidemiological information in China is not well characterized.

Objectives: To determine the NoV prevalence and genotypes associated with pediatric symptomatic and asymptomatic infections of acute gastroenteritis at the Xi'an Children's Hospital in Xi'an, China.

Study design: 201 fecal specimens were collected from hospitalized young children with diarrhea (symptomatic infection), and 53 fecal specimens were collected from gender and age matched hospitalized children without diarrhea (asymptomatic infection) between March 2009 and May 2010. NoV TaqMan real-time RT-PCR, conventional RT-PCR, and sequencing analysis were used to determine the NoV infection rates, circulating NoV genotypes, and predominant NoV variants.

Results: NoVs were detected in 19.9% (40/201) of children with symptomatic infection and in 35.8% (19/53) of children with asymptomatic infection. Only NoV GII.3 and GII.4 strains were identified, and the GII.4 sequences were similar to the 2006b variant.

Conclusions: NoVs infection appeared to be high in hospitalized children in China, indicating that NoV vaccination is urgently needed. In addition, asymptomatic NoV infection tends to be an important reservoir for NoVs transmission, and a surveillance network of NoV infections should be established to monitor the trends of NoV molecular evolution in China.

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TABLE OF CONTENTS

1. INTRODUCTION **1**

2. LITERATURE REVIEW **4**

2.1 Pathogens that Cause Acute Diarrhea 4

2.1.1.1 *Campylobacter Bacteria* 4

2.1.1.2 *Shigella Bacteria* 4

2.1.1.3 *Salmonella Enteritidis Bacteria* 6

2.1.1.4 *Aeromonas and Plesiomonas* 6

2.1.1.5 *Vibrio Cholera and Vibrio Parahaemolyticus* 7

2.1.1.6 *Eschericha coli* 8

2.1.1.7 *Clostridium difficile* 9

2.1.2 *Parasite* 10

2.1.2.1 *Giardia Parasite* 10

2.1.2.2 *Cryptosporidium parasite* 11

2.1.3 Enteric Viruses *11*

2.1.3.1 Rotavirus 11

2.1.3.2 Adenovirus 13

2.1.3.3 Astrovirus 13

2.1.3.4 Human calicivirus 14

2.2 Laboratory Diagnosis for Norovirus 17

2.2.1 Electron microscope (EM) *18*

2.2.2 Reverse Transcription PCR (RT-PCR) *18*

2.2.3 Quantitative real-time RT-PCR *19*

2.2.4 Enzyme immunoassay (EIA) *19*

3. MATERIAL AND METHODS **19**

3.1 Study Population and Study Design 20

3.2 Clinical samples Collection and Laboratory Methods 21

3.2.1 Stool sample collection *21*

3.2.2 Norovirus RNA extraction *21*

3.2.3 Norovirus detection by TaqMan Real-time RT-PCR *22*

3.2.4 Positive Norovirus amplification by conventional RT-PCR *23*

3.2.5 Strategy for determining the full-length GII.4 capsid sequence *24*

3.2.6 Electrophoresis and sequencing *25*

3.2.7 Phylogenetic analysis *26*

3.2.8 Statistical and phylogenetic analytical methods *26*

3.3 Results 27

3.3.1 Epidemiological characteristics of study population *27*

3.3.2 Norovirus detection using RT-qPCR *27*

3.3.3 Amplification and sequencing of the GII positive samples using the conventional RT-PCR.........28
3.3.4 Amplification of the fragment 2 and fragment 3 in the GII.4 capsid region................................31
3.3.5 Subclustering of GII.4 variants using partial and full-length capsid sequences...........................32

4. DISCUSSION, CONCLUSION AND RECOMMENDATION 35

4.1 DISCUSSION 35
4.2 CONCLUSION..38
4.3 RECOMMENDATION 39

REFERENCE 40

1. Introduction

Diarrhea still has been a public health hazard throughout the world, and diarrheal disease has also been a major cause of morbidity and mortality among children although advances in causative pathogens of diarrhea disease and diagnostic methods.[1,2] Diarrheal disease is the second leading cause after pneumonia in children under five years of age, and it is responsible for the death of approximately 1.5 million children each year in the world in 2004. Of these 1.5 million children, 80% were under two years old.[3] In developing countries, each child under 5 years old experiences 3 to 9 diarrheal episodes yearly.[4] On the contrary, in developed countries, each child has 2 diarrheal episodes annually. In the United States, the number of diarrheal cases every year estimates from 25 million to 99 million, resulting in about 2.2 million hospital visits. Additionally, diarrhea disease causes approximately 200,000 hospitalizations and 400 deaths in children less than 5 years old because of their susceptibility to diarrheal disease as well as other epidemiologic and immunologic reasons.[5] In China, the death rate among children under five years due to diarrheal disease is 3 per 1,000 children in 2000, and accounts for approximately one-tenth of morbidity rate for the under five population (WHO 2007).

Diarrhea is defined as three or more loose or watery stools per day. Acute diarrhea usually lasts one or two days, and goes away on its own limitation without special treatment. Prolonged diarrhea more than two days may be a sign of a more serious problem, and proposes the risk of dehydration and need intensive treatment. Severe diarrhea like persisting and chronic diarrhea may lead to more serious problems, and may be life-threatening, particularly in young children and people who have basic diseases or

impaired immunity. Diarrhea can be accompanied by the symptoms of abdominal pain, bloating, nausea, and vomiting, as well as other symptoms, such as fever, loss of appetite, cramping, dehydration and weight loss.[2,5,6] There are many different types of pathogens that cause diarrhea, such as bacteria, viruses, and parasites, usually transmitted through the fecal-oral route. Many risk factors contribute to diarrhea disease, such as improper disposal of feces, feces contact, and lack of proper hand washing after defecation. Particularly in developing countries, due to poor sanitation and hygiene, and low economy, foodborne and waterborne diarrhea outbreaks are common. While in developed countries, diarrhea outbreaks more often contribute to contaminated water and person-to-person contact.

Several hospital-based pediatric diarrhea studies have been performed in China. However, all of these studies have only focused on laboratory detection of viral pathogens and have not reported the clinical and epidemiological characteristics of viral infections in young children. In addition, several norovirus (NoV) studies in young children from other parts of the world have indicated that not all pediatric infections with NoVs result in clinical symptoms and asymptomatic NoV infection is common in young children. It is unknown whether pediatric asymptomatic infection of NoVs occurs in China and what's the rate of asymptomatic NoV excretion and the genetic diversity of NoVs.

My research objectives:

I. To determine the prevalence of norovirus infection among hospitalized children under 5 years old with diarrhea (symptomatic infection) and without diarrhea (asymptomatic infection) in Xi'an, China

II. To identify NoV genogroups and genotypes in children with symptomatic and asymptomatic NoV infections.

III. To determine partial and full-length capsid sequences in children infected with GII.4 genotypes and perform phylogenetic analysis using partial and full-length GII.4 capsid sequences.

2. Literature Review

2.1 Pathogens that Cause Acute Diarrhea

2.1.1 *Bacteria*

There are many different types of bacteria that can cause acute bacterial diarrhea in children. *Salmonella, Shigella, and Campylobacter* are the most common bacterial organisms. Because the agents could produce enterotoxins that will be responsible for the pathogenesis of diarrheal disease, the patients could have an inflammatory diarrhea with fever.

2.1.1.1 *Campylobacter Bacteria*

Campylobacter bacteria can cause acute bacterial diarrhea among children and young adults during the summer. Generally, *Campylobacter bacteria* can be transmitted via consumption of contaminated food, water, raw milk, or raw and undercooked meat. *Campylobacter jejuni* is commonly recognized as the most common cause of acute bacterial diarrhea in both developed and developing countries. It is estimated that almost 400 million cases in the world and 1.5 million cases in the United States are caused by *Campylobacter jejuni* each year.[7] At the same time, *Campylobacter* is the second cause of travelers' acute bacterial diarrhea followed by *Enterotoxigenic E. coli* (ETEC). A number of media that can be used to isolate the agents, such as MacConkey (MAC), Salmonella-Shigella (SS), eosin-methylene blue (EMB), and xylose-lysine-deoxycholate (XLD) agars.[8]

2.1.1.2 *Shigella Bacteria*

Shigella bacteria can cause acute diarrhea, also called shigellosis, which could commonly spread in families, hospitals, and child-care centers in children from 2 to 4

years old. Shigellosis is endemic in the world, especially among children under 5 years old in developing countries. It is estimated that there are about 91 million *Shigella* episodes and 414,000 deaths in Asia each year, and about 165 million cases of *Shigella* diarrhea occurs annually worldwide. Particularly, approximately 99% of *shigella* diarrheal disease occurs in the developing countries. Annually about 1.1 million people die from *shigella* infectious diseases, as well as 60% of whom are children less than 5 years old.[9] In developed countries, about 500,000 shigellosis cases occur among travelers and military each year.[10] The acute diarrhea caused by *shigella* usually accompany by abdominal pain, mucoid bloody stools, tenesmus, and fever. The transmission of *shigella* may occur through contaminated food or water, and person to person contact. *Shegella* bacteria are comprised of four species including *Shigella flexneri, Shigella dysenteriae, Shigella sonnei and Shigella boydii. S. sonnei* is the most common agent of *shigellosis* cases in developed countries and it is responsible for about 77% *shigellosis* cases in developed countries, accounting for about 15% *shigellosis* cases in developing countries. *S. flexneri* can cause endemic acute shigellosis in the developing countries, and it is also one of the most common agents causing shigellosis worldwide, about 60% of *shigellosis* is associated with *S. flexneri. S. bodyii* is a minority of spice, which occasionally cause endemic *shigelosis* worldwide.[8] *Shigella* can destroy the mucosal cells by producing elaborate cytotoxins. For example, the type I of *S. dysenteriae* can produce a kind of exotoxin. Selective and nonselective media are usually used to diagnose *shigellosis*, such as SS, HE, XLD, MAC agars.[2]

2.1.1.3 *Salmonella Enteritidis Bacteria*

Salmonella enteritidis bacteria can contaminate raw or undercooked chicken, eggs, and are the major cause of food poisoning in the summer. *Salmonella* bacteria has many different serotypes, approximately 2,501 identified.[11] Almost all the serotypes of *Salmonella* bacteria can cause acute bacterial diarrhea in humans. There are different reservoirs (animal hosts) for Salmonella bacteria. Some serotypes have single animal host; however, others have multiple animal hosts. For example, *Salmonella Dublin* is only found in cattle and *Salmonella Choleraesuis* is only reported in pigs. Particularly, there are two serotypes, *Salmonella Typhimurium* and *Salmonella Enteriditis*, which can transmit from animal hosts to human so that they can cause acute diarrhea. Generally, although most diarrheas caused by *Salmonella* bacteria are not severe, some cases are life-threatening, and need treatment, especially in children.

2.1.1.4 *Aeromonas and Plesiomonas*

Aeromonas have been recognized as an important agent that can cause acute bacterial diarrhea in children under 5 years old worldwide. *Aeromonas* have many species isolated from stools. There are some substances produced by *Aeromonas*, such as *cytotoxins, enterotoxins and hemolysins*, which are related to acute diarrhea. *Aeromonas* can be transmitted to humans via consumption of contaminated food, water and seafood. The acute diarrhea caused by *Aeromonas* can be accompanied with vomiting, abdominal pain, and fever. Generally, the diarrheal disease is not severe, and is self-limited, but severe disease like bloody diarrhea especially in children needs to be treated. Sheep blood agar (BA) and MAC agar can be used to isolate and grow Aeromonas.[2,8]

Plesiomonas shigelloides have been recognized as a cause of acute bacterial diarrhea, which may be transmitted through contaminated food, water, seafood and meats.[12] *Plesiomonas shigelloides* also can be isolated by sheep blood agar (BA) and MAC agar.

2.1.1.5 *Vibrio Cholera and Vibrio Parahaemolyticus*

Vibrio cholerae has been recognized as one major cause of epidemic acute bacterial diarrhea in Asia, Africa, and central and southern America. Currently there are only serogroup O1 and newly emerged serogroup O139 of *Vibrio cholera* that are related with epidemic cholera. The serogroup O1 of Vibrio cholera also can be classified into two biotypes, El Tor and classical. The El Tor biotype is mainly contributed to almost all the cholera disease worldwide.[2,8] *V. cholera* can produce an enterotoxin that may lead to watery diarrhea, vomiting, abdominal pain, fever, circulatory collapse, and poisoning shock. If the patient doesn't receive effective treatments, the situation of the patient may get worse quickly within several hours.[13] In general laboratory, phase-contrast or dark-field microscopy can be used to detect *Vibrio cholera* from stool specimens.[14] Because serogroup O1 of *Vibrio cholera* has two serotypes, *Inaba and Ogawa*. Immobilizing motile *Vibrio cholera* with serotype-specific *Inaba* or *Ogawa antisera* can be used to diagnose *Vibrio cholera* rapidly.[13] However, the serogroup O139 of *Vibrio cholera* may be detected by culturing stool or TCBS selective agar. There are some limitations in detection of *Vibrio cholera* by conventional biochemical and serologic methods. Because of these limitations, the rapid methods to identify *Vibrio cholera* from the stool specimens by AP-labeled, cytotoxin-specific oligonucleotide are proposed.[2]

2.1.1.6 *Eschericha coli*

There are five different types of diarrheagenic *Escherichia coli* including *Enterotoxigenic E. coli* (ETEC), *Enterohemorrhagic E.coli* (EHEC), *enteropathogenic E. coli* (EPEC), *Enteroinvasive Ecoli* (EIEC), and *Enteroaggregative E. coli* (EAEC).[2] *E. coli* is the major cause of acute bacterial diarrhea in the world, particularly in developing countries. It is also an important cause of traveler's diarrhea. *E. coli* can usually cause acute diarrhea among children through fecal-oral route, such as contaminated food or water, undercooked meats, unwashed fruit and vegetable. ETEC and EPEC are recognized as the important bacterial agents of diarrhea in children. In addition, ETEC is a critical cause of traveler's diarrhea in visitors from developing countries.[15,16,17] ETEC can be transmitted to human through the consumption of contaminated food or water. The infection caused by ETEC has the symptoms of watery diarrhea followed by vomiting, abdominal pain, and fever. Without effective treatments, the disease can last several days. In the bad situation, it can lead to dehydration, malnutrition and shock among children in developing countries. ETEC is known as the most commonly single enteropathogen among children under 5 years old in developing countries, which is probably responsible for approximately 280 million diarrhea episodes and more than 400,000 deaths each year.[18,19] In worldwide, ETEC are contributed to more than 20% of the persistent and severe diarrhea cases that need further treatment in hospitals. As reported, the prevalence of infection caused by ETEC was 14-23% among symptomatic children with diarrhea, as well as 8% among asymptomatic children. The incidence of ETEC may get lower in the children older than two years. However, the incidence of ETEC diarrhea is higher than that of rotavirus diarrhea in children.

The serotype O157:H7 of EHEC was firstly identified in 1982. *E. coli* O157:H7 can produce shiga-like toxin that is related to the infection and outbreak of cases, such as hemorrhagic colitis and severe hemolytic-uremic syndrome, particularly in children.[20] The medium of sorbitol-MacConkey (SMAC) agar is always used to isolate *E. coli* O157:H7because the medium agar contains 4-methylumbellifery1-beta-D-glucuronide (MUG) that can be used to detect glucuronidase that is produced by *E.coli*, but more than 90% E. coli O157:H7 lack the beta-glucuronidase enzyme. Currently there is one rapid method available to detect *E. coli* O157 by Latex agglutination.[2] There are many different types of fimbriae antigens in ETEC, such as coli surface antigens (CSs) and colonization factor antigens (CFAs). ETEC can express two types of toxins including heat-labile toxin (LT) and heat-stable toxin (ST), which can be responsible for the diarrhea. More than 50% ETEC can only secrete heat-stable toxin (ST), 25% of ETEC only secrete heat-labile toxin (LT), other 25% of ETEC may secrete both heat-stable toxin (ST) and heat-labile toxin (LT).[8]

2.1.1.7 *Clostridium difficile*

Clostridium difficile (C.difficile) is recognized as one of the most common agents of nosocomial diarrhea.[21,22] *C.difficile* is also considered as an important causal pathogen that causes antibiotic-associated diarrhea.[17] *C.difficile* is easy to be transmitted in people with immunodeficiency, such as patients with HIV, which can evolve into severe or fatal *C.difficile*. *C.difficile* can produce two kinds of exotoxins, toxin A and toxin B, which contribute to infection. Toxin A is a sort of enterotoxin, which may cause inflammation

related to other symptoms. However, toxin B is a potent cytotoxin that can damage colonic mucosa and cause infection.[23] Acute diarrhea caused by *C.difficile* is different from the diarrhea caused by other pathogens. Based on this characteristic, several rapid, sensitive and specific methods have been developed to detect the toxin A and toxin B. In terms of higher sensitivity and specificity, there are some detection kits available, like EIA and LA kits.[2] There are a number of other laboratory methods available to detect *C.difficile* depending on various different conditions. Selective agar can be used to identify *C.difficile* from stool specimens, such as cycloserine-cefoxitin-fructose agar (CCFA). Another method to detect *C.difficile* is direct detection related antigen and glutamate dehydrogenase in stool culture.

2.1.2 *Parasite*

Many different types of parasites have been identified as an important cause of diarrhea in the world, such as *Giardia, Cyclospora, Cryptosporidium, Strongyloides stercoralis*, and *Trichuris trichiura*, etc. Primarily the protozoal agents can cause most parasitic infections and diarrhea.

2.1.2.1 *Giardia Parasite*

Giardia parasite can cause infection, called *giardiasis.* It can also cause dehydrating diarrhea, especially common among children in developing countries. Approximately 20% patients infected with *Giardia parasite* have diarrhea. *Giardia* can be transmitted via fecal-oral route, and contaminate water. Diarrhea caused by *Giardia* is always accompanied with vomiting, abdominal pain, nausea, weight loss, anemia, and fever. Most cases are self limited with 2 to 4 weeks processing, but about more than 30% cases will become chronic diarrhea, even severe diarrhea.[24]

2.1.2.2 *Cryptosporidium parasite*

Cryptosporidium parasite can also cause watery diarrhea, persisting for at least 2 weeks. *Cryptosporidium parasite* has been recognized as an important pathogen of diarrheal diseases. *Cryptosporidium parasite* can be transmitted from person to person, which can be frequently detected in water. The symptoms of Cryptosporidium infection include diarrhea, vomiting, abdominal pain, nausea, anemia, weight loss, etc.[24]

The traditional detection of adult parasites and *proglottids* are still adopted in current diagnosis. The conventional method requires three stool specimens collected on the other day to improve yield. Although the method is not too expensive, and needs simple laboratory equipments, the current detection techniques provide more option to identify parasite from stool specimens more rapidly, sensitively and specifically, such as EIA.[2]

2.1.3 Enteric Viruses

Although many different enteric viruses have been known as the cause of acute viral diarrhea in children, including rotavirus, adenoviruses, caliciviruses, and astrovirus.[25,26] Among these enteric viruses, rotavirus, adenovirus, and norovirus can be directly tested in laboratory.

2.1.3.1 Rotavirus

Rotavirus (RV) has been recognized as the first cause of severe diarrhea in children in the world, annually about 3.5 million cases of diarrheal disease reported.[2] Rotavirus can be transmitted through the fecal-oral route, person to person contact and

contaminated food or water. Particularly in hospitals and child-care centers, rotavirus can cause diarrhea outbreaks during the winter and early spring. In terms of the economic burden of diarrheal disease in the world, a safe and effective rotavirus vaccine is needed to prevent rotavirus diarrheal infection, particularly in developing countries. Rotavirus vaccine is a very important and effective strategy to prevent the rotavirus diarrhea, and to reduce the disease burden. It is reported that the current vaccine recommended and adopted for infants has been confirmed to prevent approximately 75% cases of rotavirus infection.

The incidence of rotavirus infection has a seasonal peak, usually in winter. The children under 3 years are susceptible to rotavirus infection, and most of children infected with rotavirus usually have clinical symptoms. In the world, it is estimated that about 25% to 55% diarrheal cases were caused by rotavirus, even approximately 400,000 deaths due to rotavirus infection annually.[27] More than 80% of those deaths happen in developing countries. In Asia, based on the hospital surveillance, the prevalence of rotavirus is up to approximately 45% in children with diarrhea.[28] In China, rotavirus was detected in 41% of hospitalized children.[29]

Rotavirus is a member of Reoviridae family, with a 70 nm size of RNA virus. It contains three protein shells, including one inner and one outer capsid, and one internal core. There are two structural outer capsid proteins, VP7 (G protein) andVP4 (P protein), resulting in G and P serotypes. In the world, G1-G4 and G9 of VP7 G serotypes and P4, P6 and P8 of VP4 P genotypes, are the main genotypes.[30,31] One article reported that P8 G1 is the major strain worldwide, which is responsible for approximately 70% of

rotavirus infection in North America, Europe and Australia, about 30% in Asia and South America, and 23% in Africa.[32]

. Rotavirus antigens usually can be detected by EIA one week later after infection, and the virus can be continuous during the stage of the disease.[33] There are several laboratory methods available for detecting rotavirus in stool samples including PCR, Enzyme Immunoassay (EIA) and electron microscopy.

2.1.3.2 Adenovirus

Adenovirus is a double stranded DNA, medium-sized, non-enveloped icosahedral virus. At least 52 serotypes are responsible for adenovirus infection in humans. Usually adenovirus is persistent to different environmental conditions.

Adenovirus has been recognized as the most common cause of respiratory disease. It can also cause other diseases, such as acute non-bacterial diarrhea. Children are more susceptible to adenovirus infection. Adenovirus can be transmitted through fecal-oral route, person to person contact, and contaminated water.[34] Adenovirus has many serotypes, but only 40 and 41 cause non-bacterial diarrhea in young children.[26] The clinical symptoms of diarrhea caused by adenovirus are watery diarrhea lasting 5 to 14 days, abdominal pain, vomiting and low fever. Electron microscopy can be used to detect adenovirus in feces, and EIA can be used to detect adenovirus specific antigens.[2]

2.1.3.3 Astrovirus

Astrovirus belongs to the Astroviridae family, which has 2 genera subgroups, Avastrovirus (avian viruses) and Mamastrovirus (mammalian viruses). Astrovirus is a single-stranded RNA virus. The genome of astrovirus contains 3 overlapping open

reading frames (ORFs). ORF1a and ORF1b encode nonstructural proteins, such as serine protease and RNA polymerase, while ORF2 encodes the capsid precursor.[35]

Astrovirus recently has been considered as one common cause of acute viral diarrhea in children. Astrovirus has 8 serotypes, which are responsible for about 2.5% to 9% of the hospitalized cases with acute diarrhea[36] and type 1 is the most common serotype in the world.

2.1.3.4 Human calicivirus

Human caliciviruses(HucV), also called Small Round Structured Viruses, are 27–35 nm non-enveloped icosahedral viruses with a 7.2-8.3 kb poly-adenylated, positive sense, and single stranded RNA genome, containing three open reading frames(ORFs). HucV have two genera including norovirus (NoV) and sapovirus (SV). HucV have been recognized as the second most frequent etiologic agents in children and the major cause of foodborne and nosocomial infections of acute non-bacterial diarrhea. HucV can be transmitted by fecal-oral route, contaminated food, water or shellfish, person to person contact. HucV can survive in difficult cultures, such as in chlorinated water, freezing, and 60°C heating conditions.[8] HucV can be detected by electron microscopy, immunoelectron microscopy, PCR and EIAs. However, EIAs are not the routine approaches. Electron microscopy and immunoelectron microscopy are old methods for detecting viral diarrheal pathogens with low sensitivity.[2] Nowadays, PCR-based assays are the most common method for HucV diagnosis.

Human noroviruses have been recognized as the major cause of acute non-bacterial diarrhea outbreaks all over the world, and the second most frequent cause of viral diarrhea in children in developing countries. Noroviruses are also the leading cause of acute non-bacterial gastroenteritis in developed countries. It is estimated that approximately 10% of sporadic viral gastroenteritis and 45-97% of outbreaks of non-bacterial gastroenteritis are contributed to norovirus infection worldwide.[37,38,39] In the U.S, norovirus causes approximately 23 million infections every year in the U.S, accounting for about 60% of the economic burden by all enteric pathogens.[40] In China, approximately 72.6% of the gastroenteritis outbreaks and 39% of sporadic gastroenteritis cases are caused by norovirus.[41,42,43]

In general, noroviruses are transmitted mainly via fecal-oral routes, such as exposure to fecal-contaminated food, water, surfaces, and contact by person to person. The outbreaks of acute gastroenteritis caused by norovirus regularly occur in a variety of closed communities, such as schools, hospitals, restaurants, ships, nursing rooms and the military. The outbreaks of norovirus infection associated with contaminated water have been reported and documented. Foodborne norovirus outbreaks are usually associated with consumption of contaminated food, such as salad, sandwiches, ham and seafood. The most effective strategy to stop person to person transmission is to improve personal hygiene and sanitation, like handwash. Recently norovirus infection in new settings and special population like travelers, transplant patients, and others with severe immunosuppression are reported and emphasized.[44] The clinical symptoms of norovirus infection include watery diarrhea, abdominal pain, nausea, vomiting, low fever and

malaise. Most people have watery and non-bloody diarrhea. Some individuals may have low fever.[45] In general, norovirus infections are self-limited, but sometimes cause severe diarrhea in children, the elderly or the hospitalized patients. The symptoms of norovirus infection typically last one to two days, with an average of 24 hours incubation period, but sometimes norovirus infection can last longer. One study in the United Kingdom indicated that the symptoms of infection lasted more than 4 days in 40% of hospitalized patients older than 80 years.

Noroviruses contain a RNA genome that is single-stranded, positive-sense poly A tailed, approximately 7.6 kb in length. The norovirus genome contains three open-reading frames (ORFs) that encode nonstructural and viral structural proteins.[45,46, 47] The first open reading frame, ORF1 is a 5'-ORF encoding for a polyprotein(nonstructural proteins) containing six genes including RNA-dependent RNA polymerase(RdRp). ORF2 encodes a major capsid protein (structural protein) (VP1), which has a molecular mass of 58-65 kDa and forms the viral capsid. VP1 has two main distinct domains including the shell (S) domain and the protruding (P) domain, which are structurally and functionally independent. ORF3 encodes a minor (approximately 25-28KDa) structural protein (VP2).[48] Noroviruses have high sequence diversities, they can be further classified into 5 distinct genogroups (GI-GV), and each genogroup can be subdivided into genotypes based on the phylogenetic analysis of the RNA polymerase (RdRp) and the major capsid protein region. Only norovirus genogroups GI, GII and GIV can infect humans, while GIII strains only invade pigs and cows, and GV strain only infects mice. Currently, there are 8 genetic clusters in GI, 17 in GII, 2 in GIII, 1 in GIV identified.[49] Although the

variation likely involves the seasonal or regional pattern of infection, in general, GII genotypes account for approximately 80% of the outbreaks of norovirus infection in the world. Furthermore, among those outbreaks caused by NoV GII.4 (genogroupII genotype 4) is the most common virus strain, which is associated with 73.1% of outbreaks of acute gastroenteritis that occurred in 2006.[50]

Since norovirus GII.4 first emerged in 1990s, it has been responsible for the majority of outbreaks of norovirus infection.[51] Subsequently, in 1995-1996, a mutant GII.4 strain, named US95/96, emerged and accounted for approximately 55% of the outbreaks of norovirus infection in the US, and 85% of the outbreaks in the Netherlands. [52] Between 2000 and 2004, a new GII.4 variant associated with 80% of outbreaks of norovirus infection was reported both in the US (called Farmington Hills) and in Europe (called GII.4b).[53,54] In 2004, Europe, Australia and Asia reported a new GII.4 strain named Hunter.[55,56,57] In 2006, the 2004 GII.4 strain was replaced by two new predominant variants in the US (named Minerva), in Europe and Asia (named Sakai).[58,59]

In comparision to norovirus, the study and report of sapovirus is less limited. Sapoviruses mainly cause infection in infants, and occasionally cause endemic outbreaks. Acute diarrhea caused by sapovirus occurs less frequently than that caused by norovirus. Usually acute diarrhea caused by sapovirus is not severe than that caused by other enteric viruses.[60] Sapoviruses are divided into four genogroups (GI to GIV), of which only GI, GII and GIV infect humans.[61]

2.2 Laboratory Diagnosis for Norovirus

Because of the diversity of norovirus genotypes and antigens, it is challenging to develop sensitive and specific diagnostic assays. Since the discovery of norovirus by electron microscopy (EM) in 1972, and the first cloning sequencing of norovirus genome in 1990, more norovirus diagnostic techniques have been developed[46,47] such as reverse transcription polymerase chain reaction (RT-PCR), quantitative real-time Reverse Transcription PCR (RT-qPCR), and enzyme immunoassays (EIAs).

2.2.1 Electron microscope (EM)

One advantage for detecting norovirus by EM is the ability to directly visualize the virus without perceive of the etiology of the pathogen. However, the main disadvantage of EM detection is the lower sensitivity, approximately more than 10^6 particles required for EM detection. Moreover, it is difficult to process a large number of stool specimens simultaneously. In addition, EM detection is very expensive, and requires skilled personnel to perform.[62]

2.2.2 Reverse Transcription PCR (RT-PCR)

Currently, reverse transcription PCR (RT-PCR) is one of the most sensitive methods for norovirus detection based on the amplification of DNA from the viral RNA.[44] Although RT-PCR has more advantages than other techniques, there are also some limitations. For example, to detect all strains, different primer sets are needed. In addition, because of the high sensitivity, false positive results could occur due to the cross-contamination. Furthermore, RT-PCR is time-consuming, expensive, and requires specialized equipments.

2.2.3 Quantitative real-time RT-PCR

Real-time RT-PCR systems were developed for the production and quantitation of PCR amplicons using intercalating dyes or fluorescent probes or primers. Real-time RT-PCR demonstrated more sensitivity than conventional RT-PCR with at least a 1 log difference of detectable range.[63] TaqMan and SYBR green real-time RT-PCR assays are two primary quantitative diagnostic systems for NoV detection. SYBR green system uses the intercalating dye that binds to all double-stranded DNA and therefore additional melting curve analysis needs to be performed for the distinction of target product from non-specific products and primers-dimers. Unlike SYBR green real-time PCR, TaqMan quantitative method applies a fluorescently labeled, target-specific probe that is able to increase the specificity and sensitivity.

2.2.4 Enzyme immunoassay (EIA)

Enzyme immunoassays (EIAs), including antigen-detection EIA and antibody-detection EIA, have been developed and becoming the prospective detection technique of viruses. Enzyme immunoassays (EIAs) have the ability to detect specific virus particles and soluble norovirus antigens, which are more stable than virus RNA and in higher concentration. EIAs can be used in large scale epidemiological or clinical studies. Recently, commercial EIAs kits are available for norovirus detection in fecal specimens. However, the sensitivity of EIAs detection is lower than that of RT-PCR.

3. Material and Methods

3.1 Study Population and Study Design

The original epidemiological questionnaire designed and developed by Dr. Pengbo Liu in 2008 in the Rollins School of Public Health at Emory University was used in this study. The questionnaire was designed in both English and Chinese, and contained the risk factors associated with pediatric diarrheal diseases. In March 2009, a case-control study was designed and performed at the Xi'an Children's Hospital in China. The stool samples, epidemiological information and clinical data associated with diarrhea were collected from children under 5 years old clinically diagnosed as acute gastroenteritis and admitted into the Department of Digestive Diseases, which were defined as symptomatic infection in this study. The diagnosis of acute gastroenteritis was based on the criteria of at least 3 or more loose or watery stools per 24 hours and/or with vomiting, abdominal disorder, and fever, lasting for less than 7 days. Simultaneously, the stool specimens and related epidemiological information matched with age and gender were also collected from children without diarrhea and diarrhea relevant clinical symptoms, defined as asymptomatic infection, in the department of respiratory diseases or surgery at the same hospital. These stool specimens were selected as controls in this study. Parents/guardians from children with symptomatic and asymptomatic infections were asked to sign an informed consent before sample collection.

To detect noroviruses in stool samples, we performed a set of laboratory experiments and analyses: a) stool processing by Vertrell; b) Norovirus RNA extraction (Qiagen, Valencia, CA); c) NoV GI and GII broadly-reactive quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR); d) conventional RT-PCR

and gel electrophoresis; e) phylogenetic analysis (DNAStar, Mega4). The laboratory protocols have been successfully designed and developed by Dr. Pengbo Liu in the Rollins School of Public Health at Emory University. The consent and laboratory protocols have been submitted to IRB and received the Notification of Expedited Approval. The laboratory work was performed in the Molecular Clinical Research Center (MCRC) of the First Affiliated Hospital at Xi'an Jiaotong University, China.

3.2 Clinical samples Collection and Laboratory Methods

3.2.1 Stool sample collection

The parents or guardians were provided a sterile container with a spatula on the lid to collect stool samples after the parents/guardians agreed to join the study. After samples collection, all the stool specimens were stored at -80°C freezer for further laboratory detection and sequence analysis.

3.2.2 Norovirus RNA extraction

Before NoV RNA extraction, we cleaned the table and bench, turned on the UV light for at least 10 minutes, prepared the work area by laying down multiple layers of blue lab paper to catch stray stool, and labeled the 1.7 ml microcentrifuge tubes. The stool samples were removed from the -80°C freezer and thawed quickly in hood (without complete thawing). To make 500 µl 20% (vol/vol) stool suspension, a 400 µl of DNAse and RNAse-free water was added to the labeled 1.7 ml tubes, and the stool was scraped out using a sterile spatula and made the volume up to 500 µl. After the suspension was homogenized by vortex, a 500 µl vertrell was added into the 500 µl 20% stool suspension.

To monitor the cross contamination, a 500 µl of DNAse- and RNAse-free water was mixed with 500 µl of vertrell and used as an extraction control. After incubation overnight, the suspension was spun at 16,000 rpm for 15 min at 4°C. Subsequently, the QIAmp Viral RNA Mini Kit (QIAGEN, Valencia, CA) was used to extract viral RNA according to the manufacturer's instructions. Briefly, a 560 µl buffer AVL was added into a new 1.7 ml centrifuged tube, and 5.6 µl of the carrier RNA were mixed with the AVL buffer. The mixture was briefly vortexed for approximately 15 seconds. Then a 140 µl supernatant was removed from the old 1.7 ml centrifuge tube and added to the AVL and carrier RNA mixture, and incubated about 10 minutes at room temperature. After centrifuged briefly to remove any liquid from the tube, 560 µl of 100% ethanol was added to the viruses and mixed well. A 640 µl of the mixture was transferred to a Qiagen column and centrifuged for 1 min at 4°C at 13,000 rpm, and then the filtrate was discarded. After this procedure was repeated twice to filter out the remaining volume, a 500 µl wash buffer AW1 was added and centrifuged for 1 min at 4 °C at 13,000 rpm, and then a 500 µl wash buffer AW2 was added and centrifuged for 1 min at 4 °C at 13,000 rpm. The column was then placed in a new 1.7 ml tube and 50 µl of elution buffer was added. After incubation at room temperature for 5 min, the tube was spun down for 1 min and the RNA samples were obtained and kept at -80°C until assayed by RT-qPCR.

3.2.3 Norovirus detection by TaqMan Real-time RT-PCR

The extracted RNA samples were detected for noroviruses using one-step GI and GII real-time RT-PCR assays, respectively. The GI and GII primers/probes were located at the ORF1 and ORF2 junction regions.[64] The reverse transcription and

amplification reactions were performed on the platform ABI7500 Fast RT-PCR system (Applied Biosystems, Foster, CA). The GI real-time RT-PCR reaction included 25 µl PCR mixture containing 5.0 µl 5× buffer, 1.0 µl (10 mM) dNTP, 1.0 µl (l0 µM) of each primer, 0.75 µl (l0 µM) probe Ring 1 (G1), 0.25 µl (40 U/µl) RNAse inhibitor, 1 µl Qiagen RT-PCR enzyme mixture, 4.75 µl DNAse and RNAse free water, and 10 µl of 10-fold diluted RNA. The composition of GII real-time RT-PCR reaction was similar to the GI reaction and the only difference was the primers/probe (Table 1). The PCR program (for both G1 and G2) included 50°C 32 min, 95°C 15 min, 45 cycles of 95°C 15 sec and 56°C 1 min. To avoid cross-contamination, the RNA extracts and PCR amplification were performed on the different work areas. Positive and negative controls were set for each PCR run.

3.2.4 Positive Norovirus amplification by conventional RT-PCR

For the positive NoV samples detected by the real-time RT-PCR, a conventional RT-PCR was performed using the One-step RT-PCR Kit (Qiagen, Valencia, CA). The primer pair COG2F/G2SKR (Table 1) that targets the 3 prime region of the polymerase gene (RdRp) and 5 prime region of the capsid gene (VP1) of the NoV genome was used to amplify the N-terminal domain and a part of the shell domain of the VP1 region for generating a 387 bp PCR product.[65] PCR amplification was performed with 6.0 µl sample RNA in 24 µl PCR mixture containing 6.0 µl 5× buffer, 1.2 µl (10 mM) dNTP, 1.2 µl (l0 µM) of each primer (COG2F/G2SKR), 0.3 µl (40µ/µl) RNAse inhibitor, 1.2 µl Qiagen enzyme, and 12.9 µl DNAse and RNAse free water. After an initial denaturation at 50°C for 32 min at 95°C for 15 min, 35 cycles of amplification were performed on ABI7500

Fast RT-PCR system (Applied Biosystems, Foster, CA). Each cycle contains denaturation at 95°C for 20 sec, annealing at 50°C for 30 sec, and extension at 72°C for 40 sec, followed by final extension at 72°C for 7 min. Water control was set to monitor if the contamination occurred in each RT-PCR run.

3.2.5 Strategy for determining the full-length GII.4 capsid sequence

To determine the full-length capsid nucleotide sequences of the GII.4 strains in this study, three separate conventional RT-PCR assays were performed using conventional RT-PCR assays with overlapping primers and a "walking strategy" (Figure 1). The PCR products of the fragment 1 were obtained using the primer set COG2F/G2SKR and the method aforementioned above. The PCR amplicons of the fragment 2 and fragment 3 were amplified using primer set GII4-PL1/GII4-PL2 and GII4-PL3/GII4-PL4 (Table 1), respectively.

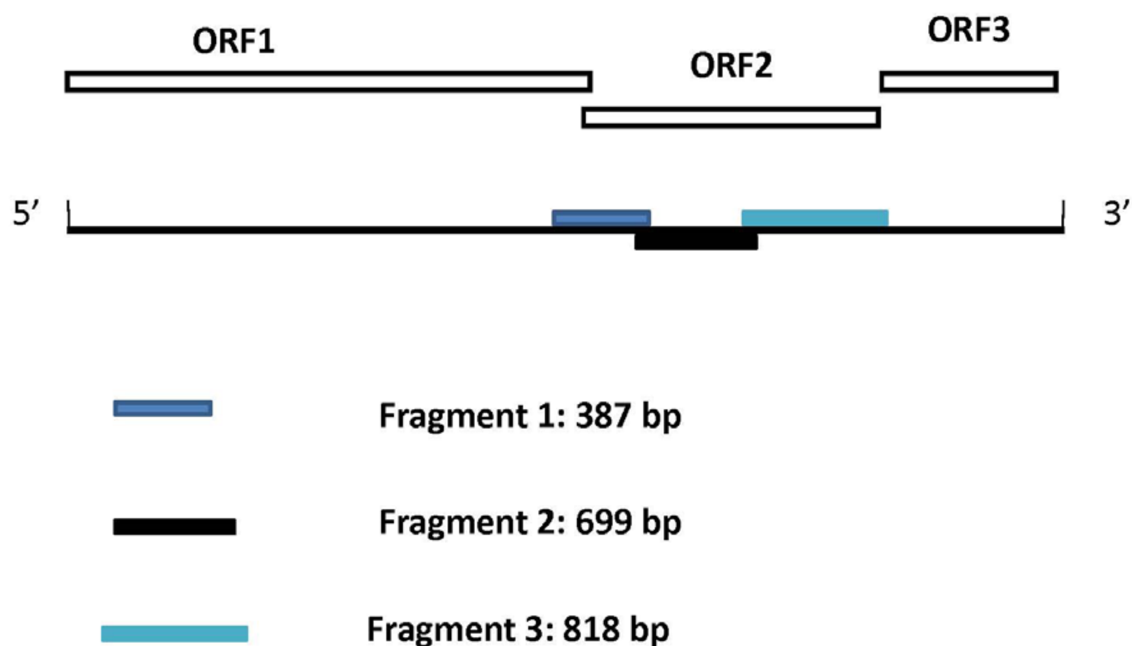

Figure 1. The "walking strategy" for determining the full-length GII.4 capsid sequences.

Table1. Primer/probe sequences used in this study

NoV	Method	Primer/ probe	Polarity	Primer sequences (5'-3')	Amplicon	Reference
GI	RT-qPCR	COG1F	+	CGYTGGATGCGNTTYCATGA	85bp	Kageyama T, et al
		COG1R	−	CTTAGACGCCATCATCATTYAC		
		RING1(A)-TP	−	FAM-AGATYGCGATCYCCTGTCCA -TAMRA		
		RING1(B)-TP	−	FAM-AGATCGCGATCTCCTGTCCA-TAMRA		
GII	RT-qPCR	COG2F	+	CARGARBCNATGTTYAGRTGGATGAG	98bp	Kageyama T, et al
		COG2R	−	TCGACGCCATCTTCATTCACA		
		RING2-TP	+	FAM-TGGGAGGGCGATCGCAATCA-TAMRA		
GII	Conventional	COG2F	+	CARGARBCNATGTTYAGRTGGATGAG	387bp	Kageyama T, et al
		G2-SKR	−	CCRCCNGCATRHCCRTTRTACAT		Kojima S, et al
GII.4	Conventional	GII4-PL1	+	GCCCTGAYTGAATCCCTAC	699bp	In-house
		GII4-PL2	−	CTGTTGGGTCATAATTGTTCC		
		GII4-PL3	+	GGGATGTCACCCACATTGCAG	818bp	
		GII4-PL4	−	AACCAAGTCCAGAGCYRAGG		

3.2.6 Electrophoresis and sequencing

The PCR products were electrophoresed in a 2% agarose gel with ethidium bromide (10 mg/ml), and then visualized under ultraviolet (UV) light. If the expected PCR amplicon was presented in the gel, the PCR product was sent to Beijing Genomics

Institute (Beijing, China) for sequencing a 387-bp PCR fregment using the forward and reverse primer pairs used in the conventional RT-PCR assay.

3.2.7 Phylogenetic analysis

All the NoV nucleotide sequences obtained in this study were edited with EditSeq program using DNASTAR software package (Madison, WI). The edited nucleotide sequences in this study and the downloaded reference sequences from the GenBank database were aligned and compared using the MegAlign programs in the DNASTAR software package (Madison, WI) and the Clustal W program implemented in the MEGA version 4 (Tamura, Dudley). The full-length GII.4 capsid nucleotide sequence from each GII.4 strain was assembled using the fragment 1, fragment 2 and fragment 3 sequences. The phylogenetic trees were constructed by the Neighbor-Joining method (MEGA V4.0) with 1,000 pseudoreplicate data sets. Phylogenetic analysis of partial capsid sequences (288 bp of the fragment 1) and complete capsid sequences (1620 bp) detected in children with symptomatic and asymptomatic infections of norovirus GII.4 variants was conducted using Neighbor-Joining method (MEGA V4.0). The following reference strains and accession numbers used in the phylogenetic analysis are: Lordsdale (X86557); Richmond (EU078406); Camberwell (AF145896); Houston (EU078407); Bochum224 (AY532125); Matsudo (AB294778); Dongen (EF126961); Beijing (EU839584); Hunter (DQ078794); Chiba (AB294780); Farmington Hills (AY502023); DenHaag (EF126956); Oxford (AY587991); Minerva (EU078417); Beijing (EU366113).

3.2.8 Statistical and phylogenetic analytical methods

The clinical and laboratory results were entered into an Excel (Microsoft, Redmond, WA) database. Statistical analysis software program, Release 9.2 (SAS Institute Inc, Cary, NC) and OpenEpi (Open Source Epidemiologic Statistics for Public Health) version 2.3 (Rollins School of Public Health, Atlanta, GA), were applied. The DNASTAR software package (Madison, WI) and MEGA version 4 (Tamura, Dudley) were performed in the phylogenetic analysis.

3.3 Results

3.3.1 Epidemiological characteristics of study population

In this study, a total of 207 children with symptomatic infection (cases) were admitted to the Department of Digestive Diseases of Xi'an Children's Hospital between March 2009 and May 2010. Of which 6 patients did not provide fecal specimens, 201 stool samples were eligible for NoV detection. Fifty-three stool samples from asymptomatic infections were collected in this study. All were eligible for testing and analysis. The mean age in the symptomatic group was 10 ± 4.9 months (range 1.5-22 months) while the mean age in the asymptomatic group was 7 ± 9 months (rang from 1 month to 50 months). Of the 40 NoV positive samples in the symptomatic children, 36 children were under 12 months old, and 20 were between 7 and 12 months old. NoV infections were detected in almost each month except for August. However, more NoV infections were identified in November (15%) and January (20%).

3.3.2 Norovirus detection using RT-qPCR

Of the 201 diarrhea stool specimens tested using the TaqMan real-time RT-PCR assays, forty (19.9%) were detected positive for NoV GII, without GI identified using

TaqMan real-time RT-PCR. Of the 53 stool specimens collected in children with asymptomatic infection, 19 (35.8%) were detected positive for NoV GII without NoV GI identified (Table 2).

Table 2. Prevalence of NoV infection in hospitalized children with symptomatic and asymptomatic infections during March 2009 and May 2010

Infection	No. specimens	NoV positive (%)	No.GI (%)	No.GII (%)
Symptomatic	201	40 (19.9)*	0	36 (18.0)*
Asymptomatic	53	19 (35.8)	0	19 (35.8)

* Of the 40 NoV positive samples, 4 samples failed to be sequenced

3.3.3 Amplification and sequencing of the GII positive samples using the conventional RT-PCR

Thirty-six (90%) of 40 NoV positive RNA samples were re-amplified using a conventional GII RT-PCR and a 388-bp fragment spanning the 3' end of the ORF1 and the 5' end of the ORF2 was determined (Figure 2). The amplicons were sequenced and analyzed with the NoV reference sequences downloaded from the GenBank database and found that all the GII positive samples belonged to either GII.4 or GII.3. Phylogenetic tree of partial capsid nucleotide sequences (288 bp) of NoV GII was constructed using 55 GII sequences including 36 cases and 19 controls from the hospitalized children with symptomatic and asymptomatic infections of NoV and 18 NoV GII reference sequences from the GenBank (Figure 3). The names of the sequences in this study started with P (patient) and C (control), and followed by the sample numbers. NoV GII reference sequences were downloaded from the GenBank with the sequence accession numbers. The scale bar represents the phylogenetic distances in units of nucleotide substitutions per site. The numbers on the branches represent the bootstrap values of the related clusters.

Bootstrap values indicate that the percentage of genogroups and genetic clusters are indicated in the 1,000 pseudoreplicate data sets.

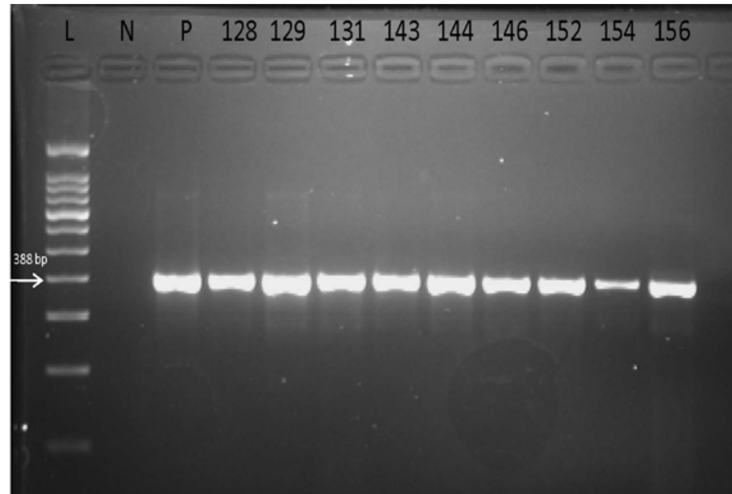

Figure 2. **Amplification of a 388-bp NoV fregment (fragment 1) using NoV conventional RT-PCR**. L: DNA ladder; N: PCR negative control; P: PCR positive control; 128,129, 131, 143, 144, 146, 152, 154 and 156 are the GII positive samples detected by previously GII RT-qPCR.

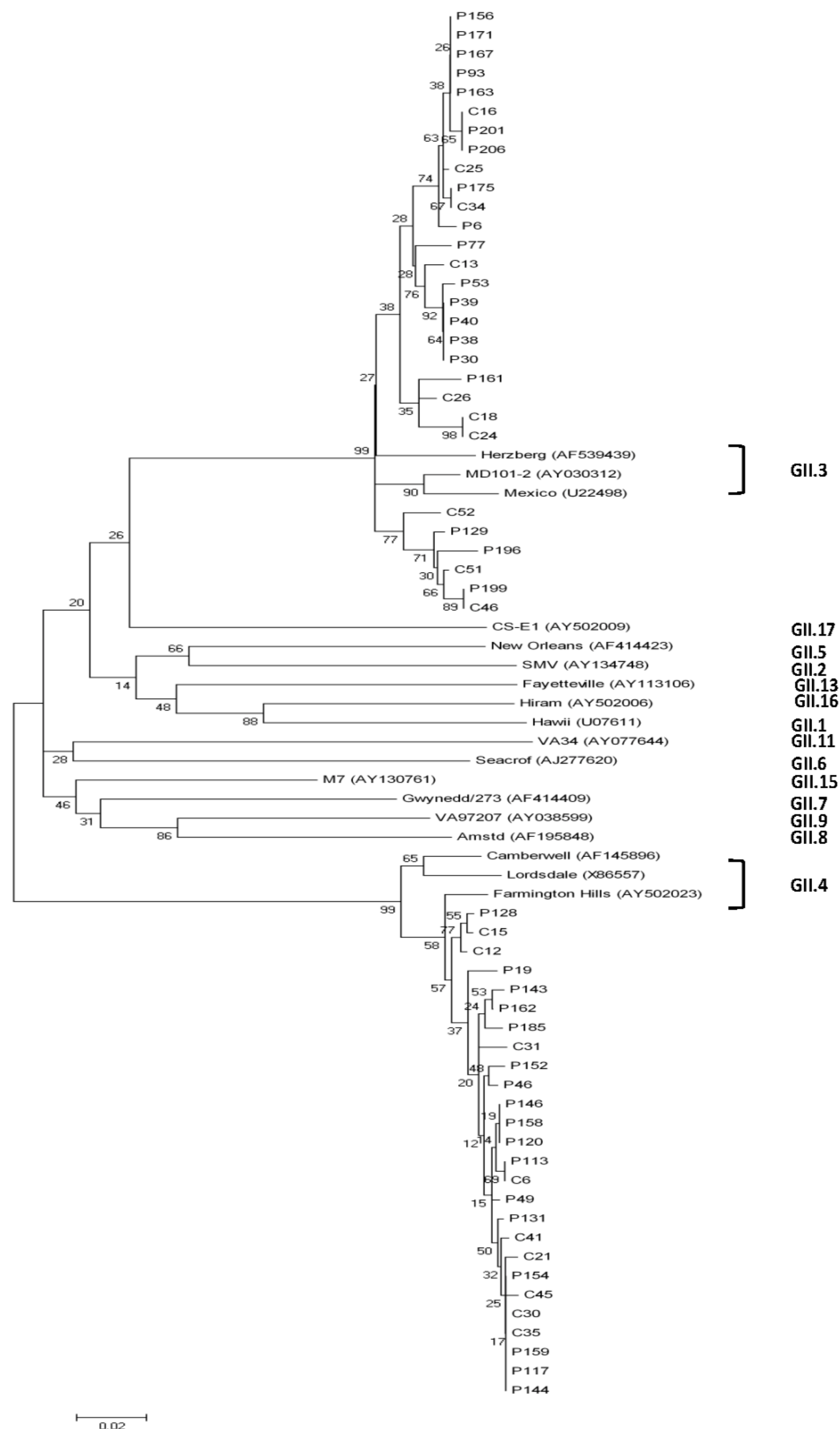

Figure 3. **Phylogenetic sequence analysis of NoV GII strains based on the capsid region VP1**. The phylogenetic tree of partial capsid nucleotide sequences (288 bp) of 55 norovirus genogroup II (NoV GII) positive samples collected in Xi'an between March 2009 and May 2010, and 18 reference sequences from the GenBank were constructed using Neighbor-Joining method.

The phylogentic analysis on the partial NoV GII capsid sequences showed that 19 (52.8%) and 17 (47.2%) NoV GII samples in symptomatic children were classified into GII.3 and GII.4, respectively. Of the 19 NoV positive samples in children with asymptomatic infection, 52.6% (10 of 19) and 47.4% (9 of 19) belonged to GII.3 and GII.4, respectively (Table 3).

Table3. Distribution of NoV genotypes in hospitalized children with diarrhea and without diarrhea during March 2009 and May 2010

Infection	No. specimens	GII	GII.3 (%)	GII.4 (%)
Symptomatic	201	36	19 (52.8)	17 (47.2)
Asymptomatic	53	19	10 (52.6)	9(47.4)

3.3.4 Amplification of the fragment 2 and fragment 3 in the GII.4 capsid region

To determine the full-length capsid nucleotide sequences of GII.4 strains, two additional PCR fragments within the GII.4 ORF2 region were amplified with overlapping primers. A 699 bp fragment 2 and a 813 bp fragment 3 (Figure 4) were obtained from 18 GII.4 positive samples (10 from symptomatic and 8 from asymptomatic infections) previously analyzed by RT-PCR amplification (fragment 1) and partial capsid sequencing.

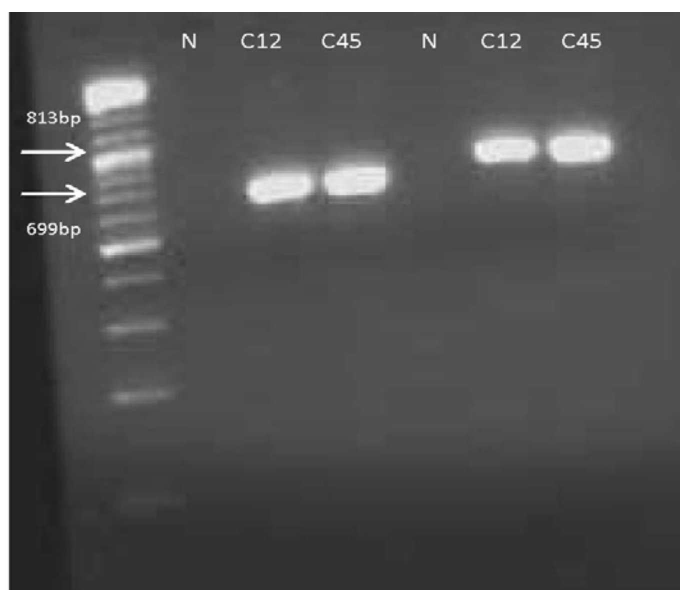

Figure 4: **Amplification of a 699-bp (fragment 2) and a 813-bp (fragment 3) fragments at the NoV capsid region using NoV conventional RT-PCR**. L: DNA ladder; N: PCR negative control; C12 and C45 are the previous RT-qPCR detected GII positive samples.

3.3.5 Subclustering of GII.4 variants using partial and full-length capsid sequences

The phylogenetic trees were constructed using partial GII.4 capsid sequences (288 bp) and full-length GII.4 capsid nucleotide sequences, assembled by three partial capsid fragments (fragment 1, 2 and 3) from 18 children with symptomatic or asymptomatic NoV GII.4 infections. Fifteen GII.4 reference sequences were downloaded from the GenBank representing five GII.4 clusters with each 3 representative sequences: (1) <1995 cluster; (2) 1995-2002 cluster; (3) 2002-2004 cluster; (4) 2004-2006 cluster; and (5) 2006 cluster. Phylogenetic sequence analysis indicated that all the NoV GII.4 variants in this study belonged to 2006b GII.4 cluster (Figure 5) using either partial GII.4 sequences or full-length sequences. Partial and full-length capsid sequences showed no significant difference for GII.4 subclustering analysis (Figure 5 and Figure 6).

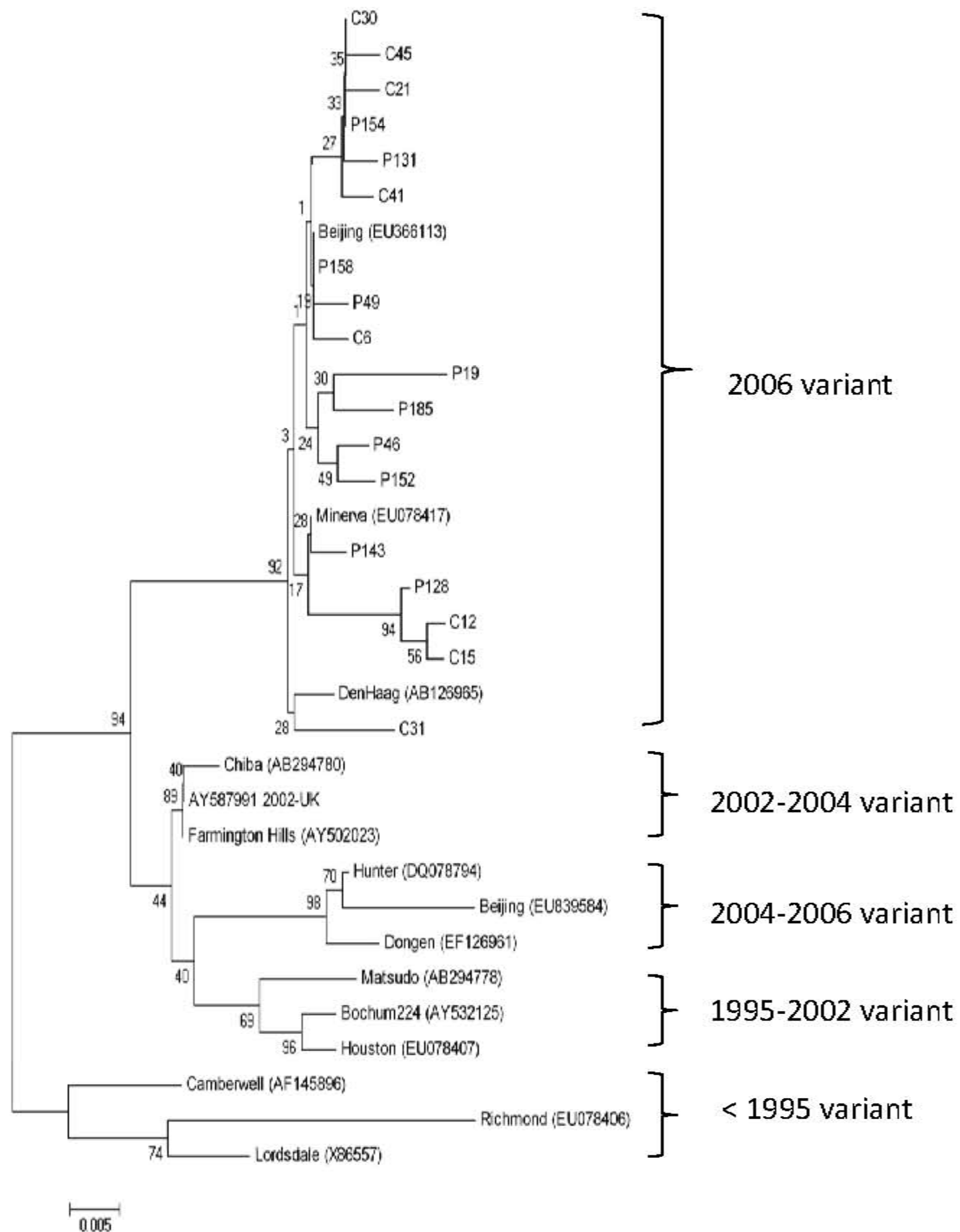

Figure 5. The phylogenetic tree of the partial capsid nucleotide sequences (288 bp) was constructed using the neighbor-jointing method based on the 18 fecal samples in the hospitalized children with symptomatic and asymptomatic infections of NoV GII.4 variants, Xi'an, China during March 2009 and May 2010, and the NoV GII.4 reference strains.

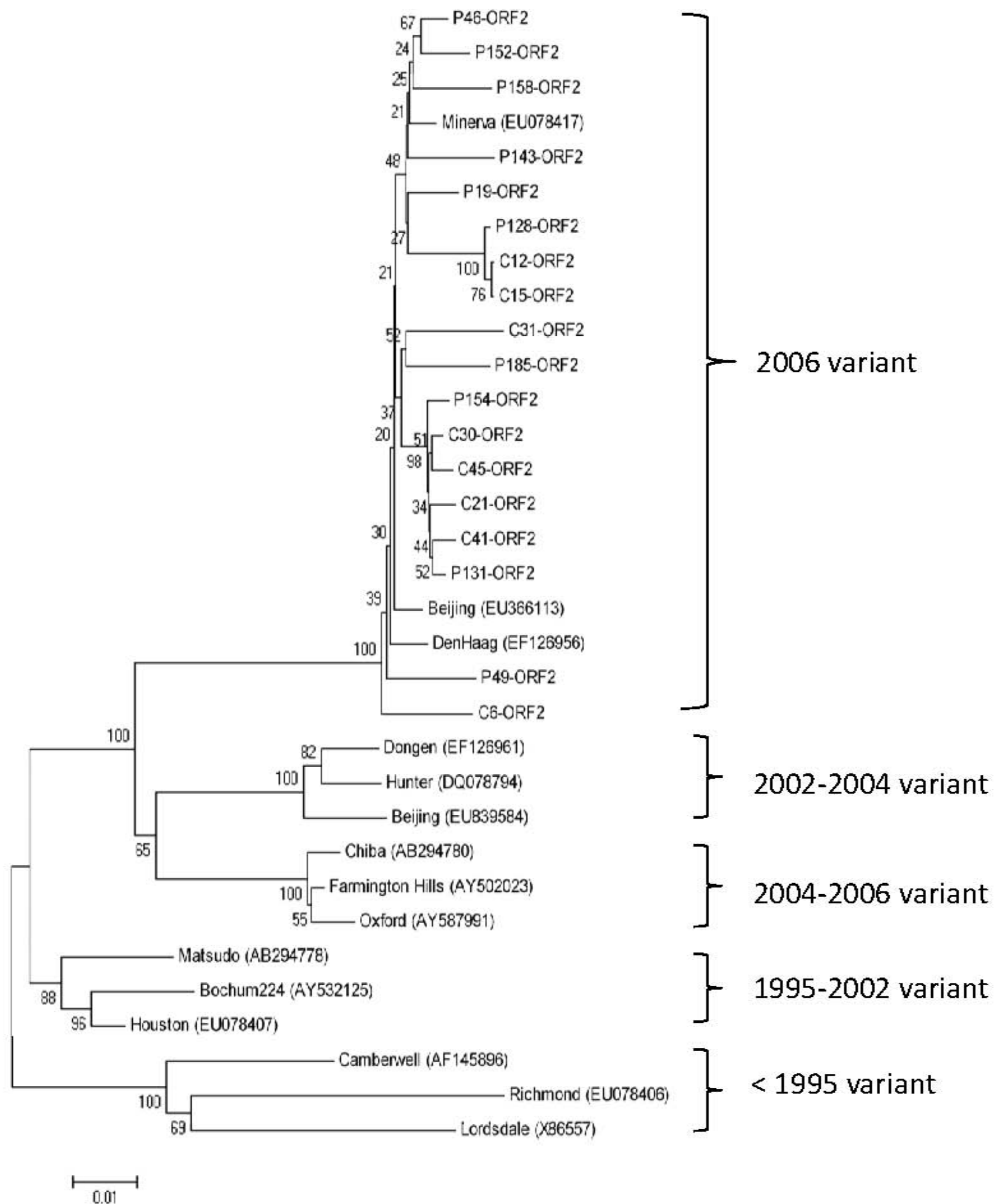

Figure 6. The phylogenetic tree of the full-length capsid nucleotide sequences (1620 bp) was constructed using the neighbor-jointing method based on the 18 fecal samples in the hospitalized children with symptomatic and asymptomatic infections of NoV GII.4 variants, Xi'an, China during March 2009 and May 2010, and the NoV GII.4 complete capsid reference sequences.

4. Discussion, Conclusion and Recommendation

4.1 Discussion

In this study, a total of 254 fecal samples were tested, fifty-nine (23.2%) were tested NoV positive in both symptomatic and asymptomatic children using the NoV real time RT-PCR. Forty (19.9%) of 201 stool specimens from children with symptomatic infection were detected positive for norovirus while 19 (35.8%) of 53 children with asymptomatic infection were detected positive for NoV. Overall, the NoV infection rate in this study is similar to the results in Guangzhou, China showing 23.9% NoV postitive rate in children from 2003 to 2006.[66] However, the NoV infection rate in this study is higher than other pediatric studies in China.[67,68,69] This discordance suggests that geography, climate, economics and cultures may contribute to the variable NoV infection rates in China. Of the 201 symptomatic diarrhea children in this study, most were under 2 years old, and in fact 50% were between 7 and 12 months. These findings are consistent with a previous study in Japan showing that NoVs infection greatly declines with the age of children increasing over 2 years old. Our results are also consistent with the other previous studies in Europe indicating that NoV infections were more predominant in young children less than 2 years old.[70] The high NoV infection in young children may be because that the young children during 12 and 24 months lack of antibody protection while children start acquiring immunity against NoV after over 2 years old.[71]

Interestingly, NoVs were detected in 35.8% of 53 asymptomatic children. This infection rate is statistically higher than that in children with symptomatic infection ($p=0.019$). The high NoV asymptomatic infection rate consists with one previous study

showing a 36.4% NoV positive rate in community children without diarrhea in Sao Paμlo State, Brazil.[72] Asymptomatic NoV infection was also reported in other previous studies.[73,74] The reason why asymptomatic children shed NoVs is probably due to virological factor, such as the diversity of NoV genogroups and genotypes, and/or the immunological factors causing NoV subclinical infection status. [75,76] This suggests that children under 2 years are more susceptible to NoV infection and subsequently may develop their protective immunity against symptomatic NoV infections after 2 years.

In this study, all the norovirus positive samples identified were NoV GII strains, while NoV GI was not detected. Forty (19.9%) of 201 diarrhea stool specimens tested NoV positive were further confirmed positive for NoV GII positive by conventional RT-PCR and phylogenetic sequence analysis. On the other hand, 19 (35.8%) of 53 stool specimens in asymptomatic children were also detected positive for NoV GII. As indicated in the literature review, NoV GII strains are predominantly prevalent in young children infected with NoVs in most parts of the world. For examples, one previous study in Dhaka city, Bangladesh, showed that the identified NoV sequences belonged to NoV genogroup II, and were further clustered into NoV GII.4.[77] In addition, in Vietnam and Japan, NoV GII was detected in 5.5% and 11.1% of the diarrhea children, respectively.[60,78] Comparing the infection rate (19.9%) in this study to other similar studies like in Australia (9%), Ireland (8%), and America (7.1%), the infection rate in this study was higher than those studies.[79,80,81]

In this study, the partial and full-length capsid nucleotide sequence analysis showed that NoV GII strains were explicitly clustered into two distinct GII genotypes,

GII.3 and GII.4), respectively. Twenty-six (47.3%) of 55 NoV GII strains belonged to the GII.4 genotype and 29 (52.7%) of 55 NoV GII strains belonged to GII.3 genotype. Previous studies indicated that NoV GII.4 was the predominant genotype causing acute gastroenteritis outbreaks and sporadic cases of gastroenteritis worldwide.[74,82] In this study, we found that the NoV GII.4 genotype was also circulating among children under 2 years old in Xi'an, China. As shown in Figure 2, the further phylogenetic sequence analysis indicated that all the NoV GII.4 in this study belonged to 2006b strain of GII.4, which was first detected in Spain in 2005, and subsequently became one major dominant strain in different areas of the world.[69] Interestingly, several previous studies found that the NoV GII.4 variants can suddenly appear and disappear in one region. In Japan, the NoV GII.4 was the most common genotype before 2004, but during 2004 and 2005, NoV GII.3 became the most dominant genotype.[71] The variation always alters due to developed population immunity. NoV GII.3 genotype is also a dominant strain in some areas in the world. The prevalence of NoV GII.3 in this study was consistent with the studies in the other parts of China. GII.3 was a predominant genotype in Guangzhou and Lulong in China.[83,84] One study during 2001 and 2005 in Shanghai indicated that GII.4 was the dominant genotype, but GII.3 circulated with other genotypes during 2001 and 2002,[68] whereas during 2003 and 2005, GII.3 suddenly disappeared. One study in Beijing showed that NoV GII.4 was the dominant genotype and circulated with GII.3 during 2004 and 2007. During 2004 and 2005, Ethime/05-30 was the dominant variant, whereas during 2006 and 2007 GII.4 2006b cluster was the dominant variant. In Lanzhou, NoV GII.4 was the common major genotype, followed by GII.3 genotype, but the prevalence of GII.3 sometimes was higher than GII.4.[69,85] The NoV GII.4 identified in this study was

the 2006b variant, which suggests that new GII.4 variant has not appeared and we should pay more attention to the emergence of new GII.4 mutant in China.

In this study, we found that there was no obvious seasonal distribution of NoV infection, NoV infections occurred in all months except August, whereas more NoV infections were in November (15%) and January (20%). Our findings are consistent with one previous study in Shanghai that most of NoV infections are emerged in late autumn and in winter, which is also similar to the study in Dhaka city, Bangladesh. However, it is different from one study that NoV infections were prevalent at the end of the rainy reason and the first half of dry season in Ho Chi Minh city, Vietnam.[61,68,77,86]

4.2 Conclusion

In summary, this study indicates that NoV is the common cause of acute gastroenteritis in young children. This study also suggests that the predominant strains and genotypes of NoV infection in hospitalized children less than 2 years old associated with acute gastroenteritis in Xi'an, China are mainly GII.4 variant. In addition, we further find that the GII.4 strains identified in Xi'an belongs to 2006b variant, which is consistent with the study in Japan, but different from the studies in the USA and Europe.[87] Therefore, further investigation of the GII.4 evolution and mutation should be focused on to better understand the probability of frequent global pandemics of NoV GII.4 and improve the global surveillance network of NoV infection in order to aid the effective control and prevention of future NoV pandemics. At the same time, since NoV is extremely contagious, shows a long shedding time, and lack of long-term immunity, the effective NoV vaccines and evaluation of vaccination are definitely required.[69] In addition, due to

the high asymptomatic infection in young children, these children tend to be the potential reservoir of NoV transmission. The NoV asymptomatic infection should be paid more attention in future prevention strategies.

4.3 Recommendation

In summary, our findings align with previous etiological studies of pediatric acute gastroenteritis, and provide important insights on norovirus study. First, as the second most prevalent enteric virus after rotavirus in pediatric diarrhea, more attention should be paid to NoV clinical diagnosis, treatment, and prevention in China. Second, since rotavirus vaccine has been successfully developed and commercialized, NoV is a logic target for vaccine development. Third, with high prevalence of asymptomatic NoV infections in young children, future studies are needed to understand the importance of asymptomatic infections acting as the potential reservoir of viral transmission in nurseries, schools, and childcare centers.

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