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ORIGINAL ARTICLE

Prevalence and molecular characterization of parechovirus A in children with acute gastroenteritis in Shenzhen, 2016–2018

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Abstract

Parechovirus A (PeV-A), which causes a wide variety of diseases, is prevalent among young children. However, little is currently known about PeV-A infections in children with acute gastroenteritis in mainland China. In this study, we investigated the molecular epidemiology of acute gastroenteritis in Shenzhen, southern China, with an emphasis on PeV-A infections. A total of 1220 stool specimens from 1220 outpatient children under 5 years old with acute gastroenteritis were collected from January 2016 to December 2018. Viral RNA was detected by a real-time RT-PCR and PCR method. The PeV-A isolates were genotyped by sequencing the VP3/VP1 region. Of 1220 specimens, 148 (12.1%) were positive for PeV-A. The predominant genotype was PeV-A 1B (68.9%), followed by PeV-A 4 (12.2%), PeV-A 14 (6.1%), PeV-A 1A (5.4%), PeV-A 6 (2.7%), PeV-A 3 (2.7%) and PeV-A 5 (2.0%). It was found that 68.2% of PeV-A infections occurred in the summer and rainy months (June to September) in southern China. The majority of PeV-A-positive patients (97.3%) were younger than 24 months old. PeV-A coinfection with norovirus, rotavirus, astrovirus and adenovirus was found in thirty specimens (30/148, 20.3%), five specimens (5/148, 3.4%), five specimens (5/148, 3.4%), and two specimens (2/148, 1.4%), respectively. Coinfections with more than one other enteric virus were not observed in any of the PeV-A-positive specimens. Phylogenetic analysis revealed that the PeV-A isolates from Shenzhen were closely related to each other and to strains circulating in China, suggesting endemic circulation of PeV-A in China. The results of this study indicate that PeV-A is one of important pathogens of acute gastroenteritis in young children and that coinfection is a possible mode of PeV-A infection. PeV-A associated with acute gastroenteritis exhibited high genotypic diversity in Shenzhen, southern China.

Introduction

Parechovirus A (PeV-A) has a single-stranded, positive-sense RNA genome of about 7,300 nt, and belongs to the genus *Parechovirus* in the family *Picornaviridae* [1]. On the basis of VP1 sequence comparisons, 18 PeV-A genotypes (PeV-A 1 to 18) have been described to date [2–4]. Originally, the prototypes PeV-A 1 and PeV-A 2 were grouped into echo-22 and echo-23, respectively, in the genus *Enterovirus*. Later, they were assigned to the genus *Parechovirus* based on their biological properties and genome organization [5]. PeV-A transmission mainly occurs through the fecal-oral and respiratory routes. Thus, virus shedding can be detected in stool and respiratory secretion specimens [6]. PeV-A is often detected in early childhood, with 20% of children being infected within the first year of life [7, 8]. It has been shown that different PeV-A genotypes are associated with different clinical symptoms and severity of disease. PeV-A 1 and PeV-A 2 infections can cause mild

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gastroenteritis, respiratory illness and even serious diseases, such as myocarditis, meningitis, fatal neonatal infection, flaccid paralysis and Reye syndrome [9, 10]. PeV-A 3 infection is more frequently associated with severe disease such as central nervous system disease and sepsis [9, 11]. The diseases caused by PeV-A 4-6 tend to be similar to those associated with PeV-A 1 and PeV-A 2 infections [12, 13]. PeV-A 7-18 infection is relatively rare in human diseases.

In mainland China, epidemiological surveillance of PeV-A infection has been reported since 2009 [14]. Little is known about PeV-A infection in children with acute gastroenteritis in China, except for six studies [7, 15–19]. Shenzhen is located on the southern coast of China and connects mainland China and Hong Kong. The city has a subtropical climate and a high population density and mobility. This study is the first to report PeV-A infection in children with acute gastroenteritis in Shenzhen, southern China. The aim of this study is to investigate the epidemiology and genetic background of PeV-A infection in children with acute gastroenteritis in Shenzhen, China, and to evaluate the relationship between acute gastroenteritis and different genotypes of PeV-A.

Materials and methods

Collection of clinical samples

Between January 2016 and December 2018, a total of 1220 fecal samples (397 in 2016, 340 in 2017, and 483 in 2018) were collected from 1220 outpatients under 5 years old with acute gastroenteritis at four sentinel hospitals and were archived at Shenzhen Center for Disease Control and Prevention, China. Of these patients, 788 were boys and 432 were girls. Acute gastroenteritis was defined as three of more loose or looser than normal stools in a 24-hour period but excluded those with pus or blood [20]. All fecal samples were stored at -20°C without additives before testing.

RNA extraction, PeV-A detection, and VP3/VP1 amplification

Total nucleic acid was extracted from 200 µl of supernatant of a 20% stool suspension using a MagNA Pure Total Nucleic Acid Isolation Kit (Roche, Germany). A real-time RT-PCR method targeting the conserved region of the 5' untranslated region (5'UTR) [21] was applied to determine the presence of PeV-A using a Perfect Real-Time One-Step Prime Script RT-PCR Kit (TaKaRa, Japan). The PeV-A isolates were genotyped by a nested PCR targeting the VP3/VP1 junction region [22]. DNA products of about 304 bp were sequenced using the Sanger method by Shanghai Sangon Co., Ltd, China. In addition to PeV-A, fecal samples were also screened for several other diarrheal viruses, including norovirus (NoV), rotavirus (RV), astrovirus (AstV) and adenovirus (AdV), using a PCR-Fluorescence Probing Detection Kit (Shenzhen Aodong inspection & testing, China).

Sequence analysis

The nucleotide sequences of the VP3/VP1 region were annotated, aligned, and edited using BioEdit version 7.0 software [23]. Multiple sequence alignments were performed using the Clustal W program (Ibis Biosciences, CA, USA) [24]. The genotypes of PeV-A isolates were determined by a Basic Local Alignment Search Tool (BLAST) search and confirmed by phylogenetic analysis. Reference sequences were obtained from the GenBank database. Phylogenetic trees were constructed in MEGA 7.0.26 using the maximum-likelihood method with 1,000 bootstrap replicates. The nucleotide substitution model used for phylogenetic reconstruction was the Tamura 3-parameter gamma model [25].

Nucleotide sequences accession numbers

A total of 148 VP3/VP1 nucleotide sequences determined in this study have been deposited in the DDBL/ENA/GenBank database under the accession numbers MN106732-MN106767, MK641524 -MK641582, and MN218129- MN218181.

Statistical analysis

Statistically significant differences in infection rates of categorical variables were tested using Fisher's exact test, the chi-square test, and the Bonferroni method. All statistics analysis was performed with SPSS Version 22 (IBM, USA). *P*-values less than 0.05 were considered statistically significant.

Results

Epidemiology of PeV-A in Shenzhen during 2016-2018

The detection rate of PeV-A was 9.1% (36/397) in 2016, 17.4% (59/340) in 2017, and 11.0% (53/483) in 2018. Overall, the detection rate of PeV-A was 12.1% (148/1220) in the three years of the study (Table 1). Eighty-eight (7.2%, 88/1220) patients who were positive for PeV-A were boys and sixty (4.9%, 60/1220) were girls. There was no statistically significant difference between the PeV-A detection rates in male and female patients ($P = 0.164$, $\chi^2 = 1.939$) (Table 2). The rates of detection of PeV-A infection in 0- to

Table 1 The detection rate of PeV-A and other diarrhea virus among children with gastroenteritis in Shenzhen, Southern China, during 2016-2018

Year	Total tested	No. PeV-A positive (%)	No. RV positive (%)	No. NoV positive (%)	No. AdV positive (%)	No. AstV positive (%)
2016	397	36 (9.1)	65 (16.4)	70 (17.6)	9 (2.3)	9 (2.3)
2017	340	59 (17.4)	71 (20.9)	60 (17.6)	16 (4.7)	5 (1.5)
2018	483	53 (11.0)	60 (12.4)	67 (13.9)	12 (2.5)	20 (4.1)
Total	1220	148 (12.1)	196 (16.1)	197 (16.1)	37 (3.0)	34 (2.8)

PeV-A, parechovirus A; NoV, norovirus; RV, rotavirus; AdV, adenovirus; AstV, astrovirus

Table 2 Demographic characteristics of children with gastroenteritis in Shenzhen, Southern China, during 2016-2018

Characteristic	Total tested	No. PeV-A positive (%)	No. of PeV-A negative (%)	*P*-value
Year (n = 1220)				
2016	397	36 (9.1)	361 (90.9)	0.002
2017	340	59 (17.4)	281 (82.6)	
2018	483	53 (11.0)	430 (89.0)	
Gender (n = 1220)				
Males	788	88 (11.2)	700 (88.8)	0.164
Females	432	60 (13.9)	372 (86.1)	
Age (months) (n = 1220)				
0 to 6	353	54 (15.3)	299 (84.7)	<0.001
7 to 12	337	59 (17.5)	278 (82.5)	
13 to 24	328	31 (9.5)	297 (90.5)	
25 to 60	202	4 (2.0)	198 (98.0)	

6-month-old patients, 7- to 12-month-old patients, 13- to 24-month-old patients, and 25- to 60-month-old patients were 15.3% (54/353), 17.5% (59/337), 9.5% (31/328), and 2.0% (4/202), respectively. The detection rate of PeV-A in 7- to 12-month-old patients was significantly higher than in those aged 0-6 months, 13-24 months, or 25-60 months ($P < 0.001$, $\chi^2 = 34.195$) (Table 2). Almost all PeV-A-infected patients were younger than 24 months (97.3%, 144/148). A sharp seasonality was observed; the majority of PeV-A infections (89.9%, 133/148) were observed between June and November, with a peak in September (Fig. 1).

Coinfection with enteric diarrhea viruses and distribution of PeV-A genotypes

All stool samples were tested for PeV-A and four other enteric diarrhea viruses, including NoV, RV, AstV and AdV. The positive rates for NoV, RV, AdV and AstV were 16.1% (197/1220), 16.1% (196/1220), 3.0% (37/1220) and 2.8% (34/1220), respectively (Table 1). NoV and RV were the most common enteric diarrheal viruses, followed by PeV-A. Coinfection with other enteric diarrhea viruses was frequently detected in PeV-A-positive stool specimens. Altogether, forty-two (28.4%, 42/148) samples were coinfected with PeV-A and other enteric diarrhea viruses, including thirty NoV-positive samples, five RV-positive samples, two AdV-positive samples, and five AstV-positive samples. No multiple infections with two or more other enteric diarrhea viruses were found in any of the PeV-A-positive stool samples. Coinfection with NoV was observed most frequently in the PeV-A-positive stool samples (Table 3).

Seven different PeV-A genotypes, PeV-A 1A, -1B, -3, -4, -5, -6, and -14, were identified in our study during 2016-2018 (Table 4). PeV-A 1B was the most common genotype (68.9%, 102/148), followed by PeV-A 4 (12.2%, 18/148), PeV-A14 (6.1%, 9/148), PeV-A 1A (5.4%, 8/148), PeV-A 6 (2.7%, 4/148), PeV-A 3 (2.7%, 4/148) and PeV-A 5 (2.0%, 3/148). The detection rate of PeV-A in acute gastroenteritis patients in 2017 was higher than that in 2016 and in 2018 ($P = 0.002$, $\chi^2 = 2.799$). There was an upward trend in the detection rate of PeV-A 4 and PeV-A 14 from 2016 to 2018, but a downward trend was observed in the detection rate of PeV-A 1A (Table 4). The PeV-A 3, -5 and -6 strains were identified less frequently. Strains PeV-A 14 and PeV-A 6 were not identified in 2016 and 2018, respectively.

Phylogenetic analysis

A total of 148 PeV-A isolates were queried in the NCBI database using the BLAST program. Reference strains shared high sequence similarity to Shenzhen strains. Phylogenetic analysis revealed that the majority of the PeV-A isolates

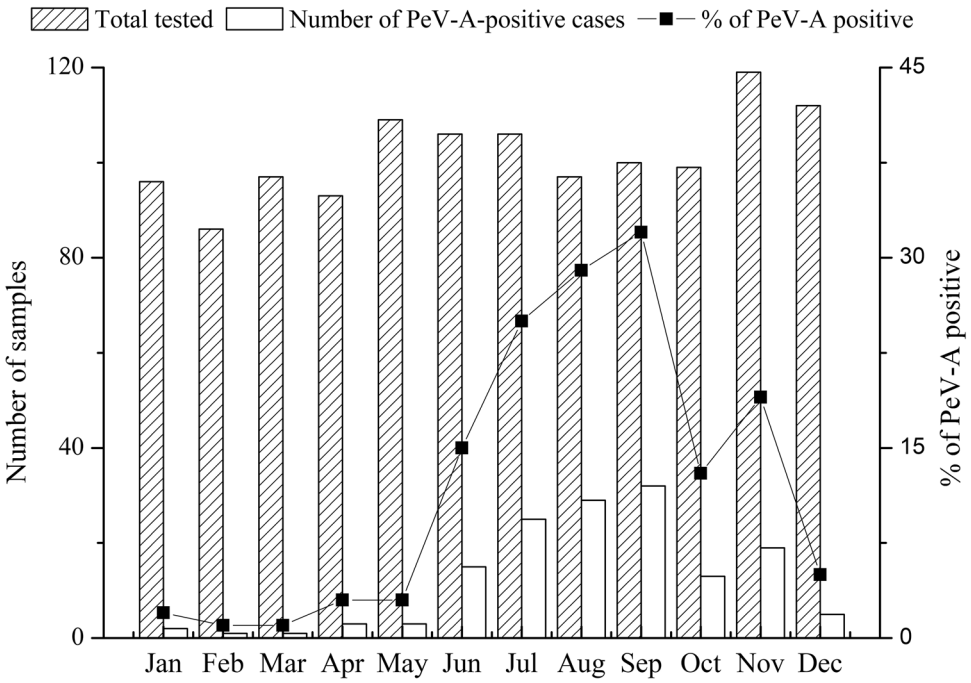

Fig. 1 Seasonality distribution of PeV-A infection among children with acute gastroenteritis in Shenzhen, southern China, 2016-2018

Table 3 Coinfection with other enteric diarrhea viruses in PeV-A-positive samples during 2016-2018

Year	Monoinfection with PeV-A	Coinfection with NoV	Coinfection with RV	Coinfection with AdV	Coinfection with AstV
2016	25	7	2	1	1
2017	37	18	2	1	1
2018	44	5	1	0	3
Total	106	30	5	2	5

PeV-A, parechovirus A; NoV, norovirus; RV, rotavirus; AdV, adenovirus; AstV, astrovirus

(68.9%, 102/148) in Shenzhen were PeV-A 1B (Fig. 2B). These PeV-A 1B strains clustered closely with strains from China (Taiwan, Shanghai, Jilin, Lanzhou, Zhejiang), Australia, Italy, Japan and South Korea circulating during 2006-2018. One hundred two PeV-A 1B Shenzhen isolates showed 82.4-99.6% nucleotide sequence identity with each other. Eighteen PeV-A 1B Shenzhen isolates from 2016-2017 were closely related to Chinese isolates from Jinlin (HQ171841) and Lanzhou (FJ763356, JQ715772), with 93.7-98.4% sequence identity, indicating endemic circulation of PeV-A 1B in Shenzhen (Fig. 2B). The eight PeV-A 1A isolates from Shenzhen were closely related to Guangzhou isolates reported in 2012-2014, with 91.4-98.8% sequence identity (Fig. 2A). These eight PeV-A 1A strains showed 76.9-97.6% nucleotide sequence identity to each other.

Three PeV-A 3 isolates (SZ510/2018, SZ567/2017 and SZ643/2016) were closely related to Chinese strains from Lanzhou, with 92.1-97.2% sequence identity, and one PeV-A 3 isolate (SZ350/2017) showed the closest relationship (99.6%) to strains from the Netherlands (Fig. 2A). These four PeV-A 3 isolates from Shenzhen shared 86.7-94.9% nucleotide sequence identity with each other.

Eighteen PeV-A 4 strains from Shenzhen clustered closely with strains from China (Lanzhou, Jilin) and Australia (Fig. 2A). These PeV-A 4 isolates in Shenzhen shared 89.9-99.6% nucleotide sequence identity with each other.

Two PeV-A 5 strains (SZ-709/2017 and SZ-586/2016) showed the closest relationship (96.5-97.6%) to Chinese strains from Guangzhou, and one PeV-A 5 (SZ-495/2018) strain was closely related to Chinese strains from Jiangsu,

Table 4 Prevalence and distribution of PeV-A genotypes in Children with acute gastroenteritis during 2016-2018

Year	Number PeV-A positive	PeV-A genotype (%)						
		PeV-A 1A	PeV-A1B	PeV-A3	PeV-A4	PeV-A5	PeV-A6	PeV-A14
2016	36	5 (13.9)	23 (63.9)	1 (2.8)	3 (8.3)	1 (2.8)	3 (8.3)	-
2017	59	2 (3.4)	43 (72.9)	2 (3.4)	6 (10.1)	1 (1.7)	1 (1.7)	4 (6.8)
2018	53	1 (1.9)	36 (67.9)	1 (1.9)	9 (17.0)	1 (1.9)	-	5 (9.4)
Total	148	8 (5.4)	102 (68.9)	4 (2.7)	18 (12.2)	3 (2.0)	4 (2.7)	9 (6.1)

Fig. 2 Phylogenetic analysis of PeV-A isolates identified in this study based on the VP3/VP1 region. The phylogenetic tree was constructed using the maximum-likelihood method with 1,000 bootstrap replicates. The nucleotide substitution model used for phylogenetic reconstruction was the Tamura 3-parameter gamma model. Only bootstrap support values >70% are shown. The PeV-A 1B clade in panel A is enlarged in panel B. The scale bar represents a genetic distance of 0.2 (A) or 0.02 (B) nucleotide substitutions per site. The symbols "filled triangle", "filled square" and "filled circle" indicate the strains from this study, collected in 2016, 2017 and 2018, respectively. The reference sequences are labeled with GenBank accession no./country/year

with 89.9% nucleotide sequence identity (Fig. 2A). The three PeV-A 5 isolates in this study showed 77.4-94.2% sequence identity to each other.

The four PeV-A 6 strains in this study showed 96.4% to 99.2% sequence identity to each other and clustered closely with PeV-A 6 strains from Australia and China (Jilin and Beijing; Fig.2A).

Eight PeV-A 14 strains from this study formed a cluster with Chinese strains from Guangzhou collected in 2013-2014, with sequence identity ranging from 92.9% to 97.2% (Fig. 2A). These eight PeV-A 14 strains showed high nucleotide sequence similarity (94.1-99.6% identity) to each other. However, a PeV-A 14 strain identified in 2018 (Shenzhen570/2018) formed a separate branch with a strain from Ghana, with low nucleotide sequence similarity (85.9% identity; Fig. 2A), and the Shenzhen570 isolates shared 76.1-78.5% sequence identity with other PeV-A 14 strains from Shenzhen.

Discussion

This study is the first to document the prevalence of PeV-A in fecal samples from children with acute gastroenteritis in Shenzhen, southern China. Some studies from China showed that the overall detection rate of PeV-A in children with diarrhea varied in different years and different regions, with a wide range from 2.0% to 55.0% [7, 15, 17–19]. The detection rate of PeV-A reported in this study also varied from year to year. The prevalence of PeV-A infection in Shenzhen was higher than that reported in Hong Kong (2.3%) [17], similar to that identified in Guangzhou (13.4%) [15], and lower than that in Lanzhou (25.3%) [7] and Shanghai (55.0%) [19]. When compared with neighboring countries, the prevalence of PeV-A infection was similar to that in India (13.9%) [26], but higher than that reported in Japan (6.8%) [27], Thailand (2.4%) [28] and South Korea (2.0%) [29]. The prevalence of PeV-A infection in 2017 was significantly higher than that in 2016 and 2018. PeV-A 14 isolates were not identified in 2016, but we cannot conclude from the data that this type was first introduced in 2017, since it might have just been absent that year. Continuous surveillance of the changing trends of PeV-A genotypes will be necessary. There was no statistical difference between the detection rates of PeV-A in female and male patients, which was in agreement with all previous studies [7, 19, 30, 31] except one [32]. The detection rate of PeV-A in 7- to 12-month-old patients was significantly higher than that in other age categories. The probable reason for this is that children begin to interact more actively with their environment and can easily be exposed to PeV-A during this period [33]. Serological studies have shown that over 90% of children are infected with PeV-A within the first two years of life [8, 34]. The current study indicated that 97.3% of children under 2 years of age with acute gastroenteritis in Shenzhen harboured PeV-A.

Seasonal variation in the rate of PeV-A infection has been reported previously, and it varies widely depending on the region of the world, the year, and the PeV-A genotypes [15, 17, 35, 36]. A distinct seasonality pattern was observed in our study, with a high frequency of PeV-A infection between July and September corresponding to the summer and rainy months in southern China. This temporal distribution differed slightly from those reported in other regions of China where the incidence of PeV-A infections peaked in July-August (Shanghai [19], Lanzhou [7], Guangzhou [15]) and in September-January (Hong Kong [17]).

Coinfection with other enteric viruses was observed in PeV-A-positive samples in our study, with norovirus being the most common coinfecting virus, which is consistent with a previous study [27]. However, some studies have found rotavirus to be the most common coinfecting virus in PeV-A-positive samples [15, 18, 37]. This difference might be due to the different populations studied and different geographical locations examined. PeV-A is regarded as an "uncertain" cause of acute gastroenteritis because other gastroenteritis-associated pathogens are also frequently found in the same stool samples. An alternative interpretation of mixed infections with gastroenteritis-associated pathogens is that PeV-A might cause synergistic effects and exacerbation of acute gastroenteritis [38]. Thus, PeV-A might be only a latent or opportunistic pathogen in the gastrointestinal tract that is activated when an individual is infected with another virus [39].

Phylogenetic analysis revealed the presence of seven different PeV-A genotypes (PeV-A 1A, PeV-A 1B, PeV-A 3, PeV-A 4, PeV-A 5, PeV-A 6 and PeV-A 14) in Shenzhen during 2016-2018. The PeV-A isolates from this study were genetically close to those circulating in Asia (China, Japan and South Korea), Europe (Germany, Italy and the Netherlands), West Africa (Ghana), and Australia, suggesting the prevalence of PeV-A worldwide. The majority of the PeV-A isolates from Shenzhen showed a high degree of nucleotide sequence similarity to each other and were closely related to Chinese strains from Guangzhou, Lanzhou, Shanghai, Jilin, Zhejiang, Jiangsu, Beijing and Taiwan, indicating endemic circulation of PeV-A in Shenzhen. PeV-A 1 was the most predominant genotype in children with acute gastroenteritis, which is in agreement with previous studies [15, 29, 39–41]. Phylogenetic analysis of the PeV-A 1 strains revealed the existence of two clusters, with the majority of the PeV-A 1 strains clustering in the PeV-A 1B clade. The predominance of PeV-A 1B isolates in Shenzhen contrasts with the reported predominance of PeV-A 1A isolates in India [26]. Generally, PeV-A 1B appears to be responsible for gastrointestinal syndrome and is often associated with acute gastroenteritis and watery diarrhea in children [32].

We also found that a high detection rate of PeV-A 4 is a notable epidemiological feature in Shenzhen. Our results was similar to those reported from the neighboring cities Hong Kong [17] and Guangzhou [15]. PeV-A 3 was rarely detected in this study. However, its prevalence was high in Asia [26, 27] and Europe [42]. It is noteworthy that PeV-A 5, PeV-A 6 and PeV-A 14, which are rarely reported genotypes, were detected unexpectedly in this study. A rapid increase in the number of PeV-A 14 strains was observed during 2016-2018. Notably, a PeV-A 14 isolate (Shenzhen570/2018) was segregated to a single branch and showed 77.3%–78.5% nucleotide sequence identity to other PeV-A 14 isolates from Shenzhen. The range of nucleotide sequence identity is less than the cutoff value described previously for genotyping of PeV-A isolates [43], which suggests the circulation of a PeV-A14 variant in Shenzhen.

In summary, we have examined the prevalence and genetic diversity of PeV-A in young children with acute gastroenteritis in Shenzhen, southern China, during 2016-2018. Coinfections with PeV-A or NoV, RV, AdV and AstV were detected in 42 of 1220 stool specimen. Routine screening for PeV-A is necessary to clarify its role in acute gastroenteritis. The epidemiology, biology and pathogenicity of PeV-A are still unclear. Therefore, the association of PeV-A infection with clinical manifestations needs to be investigated in more detail in future studies. More-detailed genotyping is also necessary to understand transmission patterns and strain-specific disease associations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Animal and human rights statement This article does not contain any studies with animals or human subjects performed by any of the authors.

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