Vital Surveillances

Vibrio parahaemolyticus O10:K4: An Emergent Serotype with Pandemic Virulence Traits as Predominant Clone Detected by Whole-Genome Sequence Analysis — Beijing Municipality, China, 2021

Ying Huang[1]; Bing Lyu[1]; Xin Zhang[1]; Yi Tian[1]; Changying Lin[1]; Lingyu Shen[1]; Hanqiu Yan[1]; Daitao Zhang[1]; Lei Jia[1]; Mei Qu[1,#]; Quanyi Wang[1,#]

ABSTRACT

Introduction: *Vibrio parahaemolyticus* (*V. parahaemolyticus*) is a common foodborne pathogen which causes gastroenteritis in humans, especially the O3:K6 pandemic clone which is still a prominent serotype in Beijing, China. In this study, we observed a novel serotype O10:K4 isolated from clinical diarrhea cases, which became the most prevalent clone in 2021.

Methods: 73 clinical isolates were collected through sentinel hospitals' surveillance in 2021. Serum agglutination testing and antimicrobial susceptibility testing were conducted. Whole genome sequencing was applied to characterize 73 *V. parahaemolyticus* strains and complete phylogenetic analysis.

Results: Seven serotypes were identified among 73 strains. O10:K4 was the most common serotype (83.6%), followed by O2:KUT, O4:KUT, and O1:KUT. Multilocus sequence typing divided the 73 isolates into 10 sequence types (STs) with ST3 as the most prevalent, which covered all O10:K4 strains. Most isolates were sensitive to common antimicrobial agents apart from colistin. All the O10:K4 isolates were positive for the thermostable direct hemolysin gene, *toxRS/new*, and *orf8*, and negative for the TDH-related hemolysin gene. The whole genome sequencing-single nucleotide polymorphism phylogenetic analysis revealed O10:K4 strains formed a main genetic lineage, which was genetically distinct from other serotypes. We also demonstrated the presence of two type III secretion system genes (T3SS1 and T3SS2) and β lactamase resistance gene *blaCARB-22* in all O10:K4 strains.

Conclusions: The study confirmed the emergence of *V. parahaemolyticus* O10:K4 possessing virulence factors similar to the O3:K6 pandemic clone, which may have enabled them to become prevalent in Beijing, China.

Vibrio parahaemolyticus (*V. parahaemolyticus*) is a halophilic bacterium that is naturally present in marine and estuarine environments and responsible for acute diarrheal illness in humans (*1*). In many Asian countries, such as China and Japan, *V. parahaemolyticus* is becoming one of the leading causes of food-borne infections (*2*).

The thermolabile hemolysin gene (*tlh*) is considered a signature molecular marker for *V. parahaemolyticus* (*1*). The virulence-associated genes of the species are thermostable direct hemolysin gene (*tdh*) and TDH-related hemolysin gene (*trh*). Up to 90% of clinical isolates of *V. parahaemolyticus* possess *tdh* and/or *trh* genes (*1*,*3*). Since 1996, O3:K6 serotype of *V. parahaemolyticus* has been prevalent in the world and causing pandemics (*1*,*4*). The pandemic clone has characteristics of tdh^+ trh^- $toxRS/new^+$ (a unique *toxRS* sequence) $orf8^{\pm}$ (the *orf8* sequence of f237 phage) (*5*–*6*). The type III secretion system (T3SS), including gene clusters T3SS1 and T3SS2, has also been shown to be associated with the pathogenicity of *V. parahaemolyticus*, which is involved in cytotoxicity to host cells and related to the enterotoxicity of *V. parahaemolyticus* (*1*,*7*).

We previously reported the serology and antimicrobial susceptibility of clinical *V. parahaemolyticus* strains, among which the O3:K6 has been the consistently dominant serotype in Beijing from 2010 to 2019 (*8*). However, an emerging serotype O10:K4 from clinical isolates was identified and became the most prevalent locally in 2021. The molecular characteristics which contributed to the survival and spread of this particular clone are rarely known. Therefore, whole genome sequence-based analysis of these isolates is of utmost importance to

elucidate their genetic characteristics, pathogenicity and transmission.

METHODS

Study Design and Population

Hospital-based active surveillance has been conducted since 2010 in Beijing, China. The sentinel hospitals affiliated with 16 different districts enrolled outpatients with acute diarrhea. The average monthly enrollment number was around 20–40 patients per district. A total of 5,337 cases were collected from January to December 2021. Enrollment was subject to obtaining informed verbal consent. All specimens were collected on the day of presentation by rectal swabs in Cary-Blair transport media and were immediately transported to the laboratory of the District Center for Disease Prevention and Control (CDC) for processing within 24 hours.

Detection of Bacteria and Serotyping

For selective enrichment of *Vibrio* spp., swabs were inoculated on peptone water containing 3% NaCl, pH 8, incubated at 37 °C overnight, then inoculated on CHROMagar *Vibrio* media (CHROMagar Co., Paris, France), and incubated for 16–24 h. After culturing, at least three suspected colonies were picked out for further identification. The systematic identification was confirmed with the VITEK 2 Compact instrument (bioMérieux, Marcyl'Etoile, France). Finally, serologic identification was performed by a slide agglutination test with 11 O (lipopolysaccharide) and 65 K (capsule) antisera (Denka Seiken Ltd., Tokyo, Japan). One serotype was defined as a unique combination of O and K serogroups.

Antimicrobial Resistance Testing

Antimicrobial susceptibility testing (AST) of *V. parahaemolyticus* strains was assessed using the broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute document (CLSI M100-S29:2019). *Escherichia coli* ATCC 25922 was included in the test as a quality control strain. Seventeen antimicrobial agents (Shanghai Xingbai Co) were used for AST: chloramphenicol, trimethoprim-sulfamethoxazole, colistin, ertapenem, meropenem, cefotaxime, ceftazidime, ceftazidime-avibactam, tetracycline, tigecycline, ciprofloxacin, nalidixic acid, aztreonam, amikacin, streptomycin, ampicillin, and ampicillin-sulbactam.

DNA Extraction and WGS

DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Quantification of extracted genomic DNA (gDNA) was determined by agarose gel electrophoresis and fluorometric analysis (Qubit 2.0). Whole genome sequencing (WGS) was conducted using an Illumina PE150 platform with 200× coverage (Novogene Technology Co., Ltd., Beijing, China). Raw sequencing data was checked for quality, trimmed, and assembled de novo into contigs. Whole genome sequencing-single nucleotide polymorphism (WGS-SNP) analysis for all draft genomes was performed using parsnp software with the reference strain sequence GCF_000196095.1 available from NCBI's genome database. The phylogenetic tree was finally visualized using the online tool iTOL (http://itol.embl.de/).

MLST, ARGs, and VGs

The genomic analysis was based on the Center for Genomic Epidemiology's web server (https://cge.cbs.dtu.dk/services/cge/). Multilocus sequence typing (MLST) 2.0 was performed using seven housekeeping genes (*dnaE*, *gyrB*, *recA*, *dtdS*, *pntA*, *pyrC*, and *tnaA*) to characterize sequence type (ST) of *V. parahaemolyticus* isolates. The new STs were submitted to PubMLST (tted to PubMLST (https://pubmlst.org/organisms/vibrio-parahaemolyticus). ResFinder 4.1 was used for screening antimicrobial resistant genes (ARGs). The virulence-associated genes (VGs) were found using virulence factor database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi).

RESULTS

Serotypes

73 out of 5,337 (1.4%) diarrheal outpatients were positive for *V. parahaemolyticus* in 2021. Serological analysis of the 73 *V. parahaemolyticus* isolates revealed a total of 7 serovars with 3 defined serotypes (O10:K4, O3:K6, and O6:K18) and 4 kinds of untypeable K antigens. O10:K4 (83.6%, 61/73) was the most common one, followed by O2:KUT (5.4%, 4/73), O4:KUT (4.1%, 3/73), O1:KUT (2.7%, 2/73) and O3:K6, O6:18, and O10:KUT each (1.4%, 1/73) (Table 1). These results indicated the emerging serotype O10:K4 had replaced O3:K6, which accounted for 67.7% of clinical isolates during the period of 2010–2019 (*8*), becoming the predominant

serotype in 2021.

Antibiotic Resistance Profile and Resistance Genes

The antimicrobial susceptibilities of 73 *V. parahaemolyticus* strains were listed in Table 2. All isolates were sensitive to the following 14 antimicrobials agents such as ampicillin-sulbactam, ceftazidime-avibactam, cefotaxime, ceftazidime, ertapenem, meropenem, amikacin, tetracycline, aztreonam, tigecycline, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole and chloramphenicol. Only 2.7% of 73 isolates were resistant to colistin and 97.3% demonstrated intermediate resistance to colistin. Additionally, the sensitivity rates of 73 isolates to ampicillin and streptomycin were 89.0% and 67.1%, respectively, and the intermediate resistance rates were 11.0% and 32.9%, respectively. The ARGs analysis showed that all 73 strains carried at least one of the 9 kinds of β lactamase resistance genes (*blaCARB-18*, *blaCARB-20*, *blaCARB-22*, *blaCARB-24*,

TABLE 1. Serotypes, ST and virulence factors of 73 clinical *V. parahaemolyticus* strains in Beijing, 2021.

Serovars	No. of isolate (s)	ST	Virulence genes		Pandemic markers	
			tdh	*trh*	*toxRS/new*	*orf8*
O10:K4	61	ST3	+	−	+	+
O3:K6	1	ST3	−	−	+	−
O6:K18	1	ST1490	−	−	+	−
O1:KUT	1	ST3	+	−	+	−
	1	ST2620	−	−	+	−
O2:KUT	4	ST2781, ST2894, ST2895, ST2896	−	−	+	−
O4:KUT	1	ST499	−	−	+	−
	2	ST2516	+	−	+	−
O10:KUT	1	ST2897	−	−	+	−

Abbreviations: ST=sequence type; *V. parahaemolyticus*=*Vibrio parahaemolyticus*.

TABLE 2. Antimicrobial susceptibility of 73 clinical *V. parahaemolyticus* strains in Beijing in 2021.

Antimicrobial class	Antimicrobial agent	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Penicillins	ampicillin	65(89.0)	8(11.0)	0
ß-Lactam/ß-lactamase inhibitor combinations	ampicillin-sulbactam	73(100.0)	0	0
	ceftazidime-avibactam	73(100.0)	0	0
Cephems	cefotaxime	73(100.0)	0	0
	ceftazidime	73(100.0)	0	0
Carbapenems	ertapenem	73(100.0)	0	0
	meropenem	73(100.0)	0	0
Aminoglycosides	amikacin	73(100.0)	0	0
	streptomycin	49(67.1)	24(32.9)	0
Macrolides	aztreonam	73(100.0)	0	0
Tetracyclines	tetracycline	73(100.0)	0	0
	tigecycline	73(100.0)	0	0
Quinolons and fluoroquinolones	nalidixic acid	73(100.0)	0	0
	ciprofloxacin	73(100.0)	0	0
Folate pathway inhibitors	trimethoprim-sulfamethoxazole	73(100.0)	0	0
Phenicols	chloramphenicol	73(100.0)	0	0
Lipopeptides	colistin	0	71(97.3)	2(2.7)

Abbreviation: *V. parahaemolyticus*=*Vibrio parahaemolyticus*.

blaCARB-29, *blaCARB-30*, *blaCARB-33*, *blaCARB-34*, and *blaCARB46*) (Figure 1). Two strains had the quinolone resistance gene *qnrC*. Interestingly, all 61 O10:K4 strains carried *blaCARB-22*.

Distribution of Virulence-associated Genes

All of the 73 strains had the *tlh* gene, but none had the *trh* gene (Figure 1). 64 isolates (87.7%) were positive for the *tdh* gene, of which 61 strains carried the *orf8* gene. The serotypes of these 64 *tdh*$^+$ strains included O10:K4 (n=61), O4:KUT (n=2), and O1:KUT (n=1) (Table 1). In addition, all 64 *tdh*$^+$ strains were pandemic clones with gene marker *tdh*$^+$ *trh*$^-$ *toxRS/new*$^+$. The other 9 *tdh*$^-$ strains belonged to serotypes O3:K6 (n=1), O6:K18 (n=1), O1:KUT (n=1), O2:KUT (n=4), O4:KUT (n=1), and O10:KUT (n=1). All 73 strains contained multivalent adhesion molecules encoding the *VP1611* gene and nearly all 39 T3SS1 genes except for *vopB* and *vscF*. The 64 *tdh*$^+$ strains carried all 25 T3SS2 genes, but the 9 *tdh*$^-$ strains were negative for the 25 T3SS2 genes (Figure 1).

MLST Analysis

A total of 73 *V. parahaemolyticus* strains were categorized into 10 STs. Four new STs (ST2894, ST2895, ST2896, and ST2897) were identified. The most frequently observed ST was ST3 (63/73, O10:K4 n=61, O3:K6 n=1, and O1:KUT n=1). The 64 pandemic strains (*tdh*$^+$ *trh*$^-$ *toxRS/new*$^+$) belonged to ST3 (O10:K4 n=61 and O1:KUT n=1) and ST2516 (O4:KUT n=2) (Table 1). All 61 O10:K4 isolates had the characteristic of *tdh*$^+$ *trh*$^-$ *toxRS/new*$^+$ *orf8*$^+$ ST3, which was also characteristic of most strains from diarrhea patients.

Phylogenetic Analysis

The phylogenetic analysis of the 73 strains was evaluated using a WGS-SNP analysis with the reference sequence GCF_000196095.1. All of the 61 O10:K4 strains with ST3 formed the main lineage

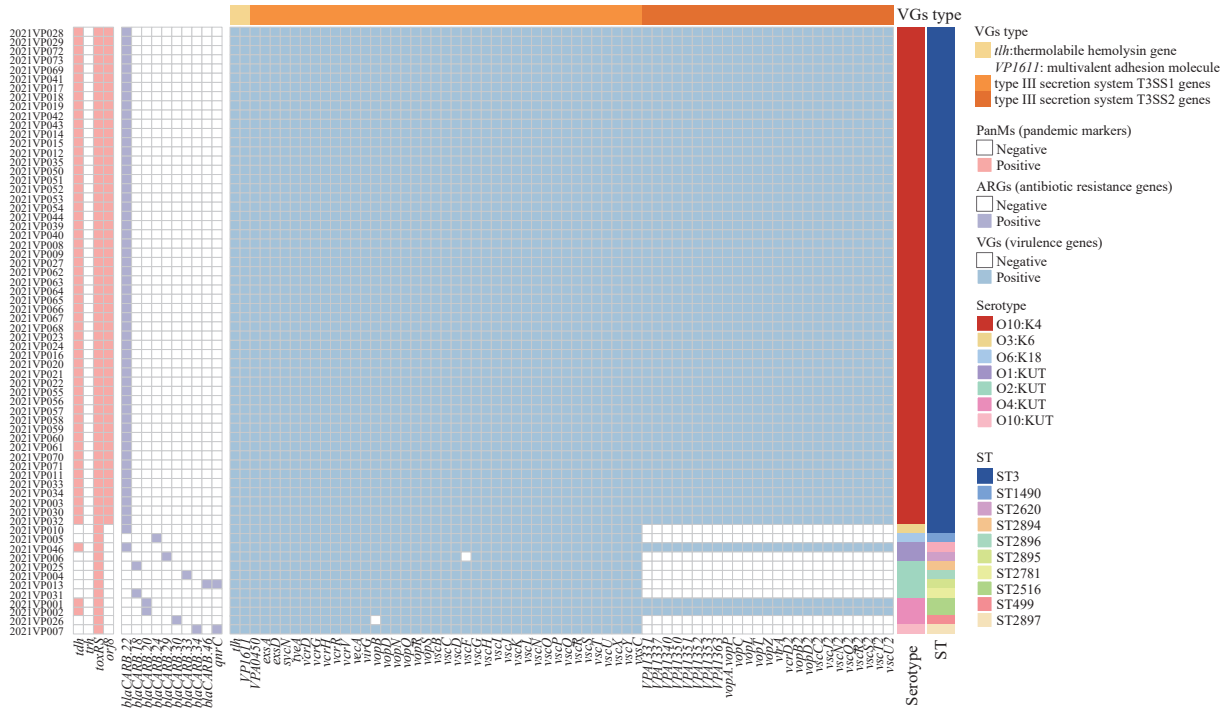

FIGURE 1. Distributions of serotype, STs, antibiotic resistance genes, virulence genes, and pandemic markers among 73 clinical *V. parahaemolyticus* strains in Beijing in 2021.
Note: The color strips indicate areas corresponding to the isolates. Pink colored cells represent the presence of pandemic markers and white cells represent the absence of the pandemic markers; Lilac colored cells represent the presence of antibiotic resistance genes and white cells represent the absence of the antibiotic resistance genes; Light blue cells represent the presence of virulence-associated genes and white cells represent the absence of the virulence-associated genes.
Abbreviations: ST=sequence type; *V. parahaemolyticus*=*Vibrio parahaemolyticus*.

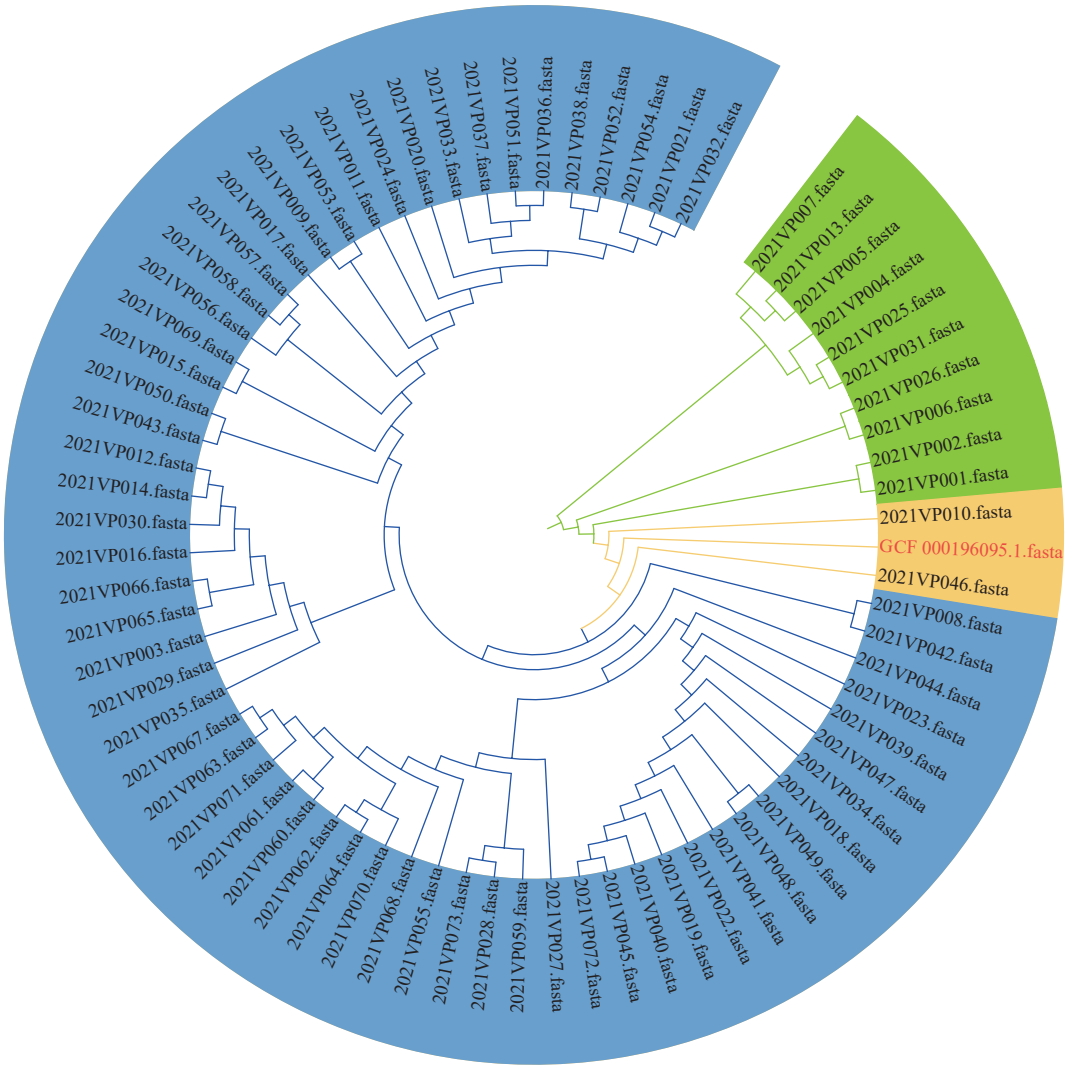

FIGURE 2. Phylogenetic tree of 73 clinical *V. parahaemolyticus* strains by WGS-SNP analysis in Beijing in 2021.
Note: The 61 genomes from O10:K4 strains with ST3 were indicated within the blue ring lineage. The two genomes from the other ST3 strains (2021VP046 belonging to O1:KUT and 2021VP010 belonging to O3:K6) were indicated within the yellow ring lineage. The other 10 genomes from 5 serotypes (O6:K18, O1:KUT, O2:KUT, O4:KUT, and O10:KUT) and 9 different STs, were indicated within the green ring lineage.
Abbreviations: *V. parahaemolyticus*=*Vibrio parahaemolyticus*; WGS=whole genome sequence; SNP=single nucleotide polymorphism; ST=sequence type.

(Figure 2), which was close to the other two ST3 strains (2021VP046 belonging to O1:KUT and 2021VP010 belonging to O3:K6). The other 10 strains belonging to 5 serotypes (O6:K18, O1:KUT, O2:KUT, O4:KUT, and O10:KUT) and 9 different STs, formed the individual branches.

DISCUSSION

V. parahaemolyticus serotype O3:K6 with pandemic markers (*tdh*$^+$ *trh*$^-$ *toxRS/new*$^+$ *orf8*$^\pm$) has been widespread in many countries including China since 1996 (*1*,*4*). In this study, only one of 73 clinical *V. parahaemolyticus* isolates was identified as O3:K6 in Beijing in 2021, which was much lower than our previous study reporting of 67.7% over the previous 10 years from 2010 to 2019 (*8*) and 48% of the clinical isolates of *V. parahaemolyticus* collected in Guangdong Province from 2007 to 2011 (*9*). Moreover, this O3:K6 isolate was neither a pandemic nor a pathogenic strain. Above all, 61 O10:K4 strains (83.6%) with pandemic traits (*tdh*$^+$ *trh*$^-$ *toxRS/new*$^+$ *orf8*$^+$) were found for the first time and became the dominant clone instead of O3:K6 in 2021. The emergence of pathogenic and pandemic *V. parahaemolyticus* O10:K4 strains presented in this

study should be a matter of concern for public health authorities, as the risk of outbreak rises. Recent studies have shown that at least 21 non-O3:K6 serotypes such as O4:K8, O4:KUT, and O3:K8 exhibited pandemic markers, and most likely originated from the same clones as O3:K6 (*4*,*10*). These findings suggest that the O and K antigen encoding loci are subject to exceptionally high rates of recombination (*11*). Serovar conversion through mutation or horizontal gene transfer of the O and K antigen encoding genes may be one means for *V. parahaemolyticus* to adapt to environmental changes and human immune responses (*12*).

In addition, this study revealed that T3SS1 genes were present in all *V. parahaemolyticus* strains and T3SS2 genes were predominantly present in the pathogenic and pandemic strains, indicating that the T3SS2 region may enhance virulence when present. Our results are consistent with the recent finding in an experimental animal model, which demonstrated that T3SS2 is necessary for pathogen colonization and the development of gastroenteritis (*13*). In this study, all the O10:K4 strains carried the entire T3SS1 and T3SS2 genes, which confirmed that the O10:K4 strains had a similar virulence as the O3:K6 pandemic clone. The 9 *tdh*$^-$ *trh*$^-$ T3SS2$^-$ strains were isolated from diarrheal patients, which also suggested that *V. parahaemolyticus* might harbor other virulence factors responsible for diarrhea. Therefore, the understanding of pathogenicity is still incomplete and it is necessary to discover more reliable predictors of virulence.

Among 10 STs analyzed in this study, ST3 was the most common one, which matched the findings of previous studies that ST3 was representative of pandemic clones on a global scale (*14*). The correlation between different serogroups and STs has been observed and some STs contained several different serogroups, such as ST3 (O1 and O3) (*9*,*15*). This phenomenon was observed in this study, that all the ST3 strains belonging to different serotypes O10:K4, O1:KUT, and O3:K6 formed a cluster, different from other STs which broke into individual branches in the phylogenetic analysis. Moreover, all the O10:K4 strains and the genetic variant O3:K6 (*tdh*$^-$ *trh*$^-$ *toxRS/new*$^+$ *orf8*$^-$) were placed in the same cluster, suggesting a possibility of transfer of the pandemic clone.

To the best of our knowledge, this was the first report of O10:K4 associated with diarrhea cases in China. The whole-genome sequence analysis indicated that it belonged to ST3 lineage which has the capacity to spread rapidly and the potential to replace native strains. However, it was unclear where this novel clone originated from and how it entered Beijing. Therefore, it is necessary to track the source of O10:K4 strains and to strengthen monitoring of their spread and epidemic trends through the continuous surveillance of *V. parahaemolyticus* in the future.

Conflicts of Interest: No conflicts of interest.

Acknowledgements: Staff of District CDCs of Beijing Municipality.

doi: 10.46234/ccdcw2022.106

[#] Corresponding authors: Mei Qu, meiqu@126.com; Quanyi Wang, bjcdcxm@126.com.

[1] Beijing Center for Disease Prevention and Control, Beijing Key Laboratory of Diagnostic and Traceability Technologies for Food Poisoning, Beijing, China.

Submitted: April 12, 2022; Accepted: May 31, 2022

REFERENCES

1. Letchumanan V, Chan KG, Lee LH. *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. Front Microbiol 2014;5:705. http://dx.doi.org/10.3389/fmicb.2014.00705.
2. Yan WX, Dai Y, Zhou YJ, Liu H, Duan SG, Han HH, et al. Risk factors for sporadic *Vibrio parahaemolyticus* gastroenteritis in east China: a matched case-control study. Epidemiol Infect 2015;143(5):1020 – 8. http://dx.doi.org/10.1017/S0950268814001599.
3. Zhang HZ, Sun SF, Shi WM, Cui L, Gu QF. Serotype, virulence, and genetic traits of foodborne and clinical *Vibrio parahaemolyticus* isolates in Shanghai, China. Foodborne Pathog Dis 2013;10(9):796 – 804. http://dx.doi.org/10.1089/fpd.2012.1378.
4. Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA. Global dissemination of *Vibrio parahaemolyticus* serotype O3: K6 and its serovariants. Clin Microbiol Rev 2007;20(1):39 – 48. http://dx.doi.org/10.1128/CMR.00025-06.
5. Laohaprertthisan V, Chowdhury A, Kongmuang U, Kalnauwakul S, Ishibashi M, Matsumoto C, et al. Prevalence and serodiversity of the pandemic clone among the clinical strains of *Vibrio parahaemolyticus* isolated in southern Thailand. Epidemiol Infect 2003;130(3):395 – 406. http://dx.doi.org/10.1017/S0950268803008458.
6. Matsumoto C, Okuda J, Ishibashi M, Iwanaga M, Garg P, Rammamurthy T, et al. Pandemic spread of an O3: K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. J Clin Microbiol 2000;38(2):578 – 85. http://dx.doi.org/10.1128/JCM.38.2.578-585.2000.
7. Noriea III NF, Johnson CN, Griffitt KJ, Grimes DJ. Distribution of type III secretion systems in *Vibrio parahaemolyticus* from the northern Gulf of Mexico. J Appl Microbiol 2010;109(3):953 – 62. http://dx.doi.org/10.1111/j.1365-2672.2010.04722.x.
8. Qu M, Tian Y, Huang Y, Liu BW, Zhang X, and Lyu B, et al. Serotype distribution and drug resistance analysis on clinical isolates of *Vibrio parahaemolyticus* in Beijing, 2010-2019. Capial J Public Health 2020;14(6):285 – 90. http://dx.doi.org/10.16760/j.cnki.sdggws.2020.06.003. (In Chinese).
9. Li BS, Luo JY, Tan HL, Ke BX, He DM, Ke CW, et al. Phenotypic and phylogenetic analysis of *Vibrio parahaemolyticus* isolates recovered from diarrhea cases in Guangdong Province, China. Int J Food Microbiol 2015;200:13 – 7. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.12.

009.
10. Velazquez-Roman J, León-Sicairos N, Flores-Villaseñor H, Villafaña-Rauda S, Canizalez-Roman A. Association of pandemic *Vibrio parahaemolyticus* O3: K6 present in the coastal environment of Northwest Mexico with cases of recurrent diarrhea between 2004 and 2010. Appl Environ Microbiol 2012;78(6):1794 – 803. http://dx.doi.org/10.1128/AEM.06953-11.
11. Gavilan RG, Zamudio ML, Martinez-Urtaza J. Molecular epidemiology and genetic variation of pathogenic *Vibrio parahaemolyticus* in Peru. PLoS Negl Trop Dis 2013;7(5):e2210. http://dx.doi.org/10.1371/journal.pntd.0002210.
12. Mahmud ZH, Neogi SB, Kassu A, Wada T, Islam MS, Nair GB, et al. Seaweeds as a reservoir for diverse *Vibrio parahaemolyticus* populations in Japan. Int J Food Microbiol 2007;118(1):92 – 6. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.05.009.
13. Ritchie JM, Rui H, Zhou X, Lida T, Kodoma T, Ito S, et al. Inflammation and disintegration of intestinal villi in an experimental model for *Vibrio parahaemolyticus*-induced diarrhea. PLoS Pathog 2012;8(3):e1002593. http://dx.doi.org/10.1371/journal.ppat.1002593.
14. Han DS, Tang H, Lu J, Wang GZ, Zhou L, Min LF, et al. Population structure of clinical *Vibrio parahaemolyticus* from 17 coastal countries, determined through multilocus sequence analysis. PLoS One 2014;9(9):e107371. http://dx.doi.org/10.1371/journal.pone.0107371.
15. Banerjee SK, Kearney AK, Nadon CA, Peterson CL, Tyler K, Bakouche L, et al. Phenotypic and genotypic characterization of Canadian clinical isolates of *Vibrio parahaemolyticus* collected from 2000 to 2009. J Clin Microbiol 2014;52(4):1081 – 8. http://dx.doi.org/10.1128/JCM.03047-13.