

Pathogenic Characteristics of and Variation in *Vibrio parahaemolyticus* Isolated from Acute Diarrhoeal Patients in Southeastern China from 2013 to 2017

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Objective: *Vibrio parahaemolyticus* is a major diarrhoea-inducing pathogen in coastal areas. In this study, we analysed the pathogenic characteristics of and variation in *V. parahaemolyticus* isolated from acute diarrhoeal patients in seven hospitals in different areas of southeastern China from 2013 to 2017.

Methods: The fecal specimens of patients with acute diarrhoea were collected. The routine microbiological test procedure combining with MALDI Biotyper microbial identification system was carried out to identify the *V. parahaemolyticus*. Serum agglutination tests, PCR for the detection of virulence-related genes and the Kirby-Bauer method to test for antimicrobial sensitivity were performed.

Results: From 2013 to 2017 in southeastern China, a total of 1220 *V. parahaemolyticus* strains were isolated from 16,504 stool specimens collected from acute diarrhoeal patients, and the annual isolation rate fluctuated between 6.1% and 8.7%. In total, 96.7% of the *V. parahaemolyticus* isolates were isolated in summer and autumn, mainly in people aged 18–44. Fifty-nine serotypes were identified, and the agglutination rate of the O antigen was 98.5%. From 2014 to 2016, the dominant serotype was O3:K6, while in 2013 and 2017, it was O4:KUT. The serotypes of O3:K6, O4:KUT, O4:K8, O3:KUT, O10:K60, O1:KUT and O1:K36 appeared every year from 2013 to 2017. O4:K6 and O4:KUT began to appear after 2014 and 2015, respectively. A total of 49.5% of the strains belonged to the pandemic group, which consisted of 26 serotypes. Most isolates were sensitive to common antibiotics, excluding ampicillin.

Conclusion: *V. parahaemolyticus* is still present at a high level in southeastern China. Although the pandemic O3:K6 serotype is predominant, new serotypes continue to emerge, especially the O4:KUT serotype, which exceeded O3:K6 in prevalence in some years. Long-term surveillance is necessary to prevent the outbreak or transmission of this pathogen.

Keywords: Acute diarrhoea, *Vibrio parahaemolyticus*, serotypes, virulence genes, antimicrobial resistance

Introduction

Diarrhoea was the eighth leading cause of death in the world in 2016, accounting for more than 1.6 million deaths.¹ *Vibrio parahaemolyticus*, which is naturally present in coastal waters, is the leading bacterial cause of gastroenteritis associated with seafood consumption. In Southeast Asian countries, such as China, Thailand and Japan, *V. parahaemolyticus* is the major pathogens for foodborne illnesses.^{2–5} Even in the United States and Europe, *V. parahaemolyticus* outbreaks are frequently reported.^{6–8}

V. parahaemolyticus is a kind of microorganism with high genetic diversity. Serological typing, pulsed field gel electrophoresis, multilocus sequence typing, and whole genome sequencing are commonly used in tracing the origin and genetic variation of *V. parahaemolyticus*. Serological typing is the most common method. *V. parahaemolyticus* can be differentiated by serotyping by the O serogroups and K serotypes. At present, there are 11 O and 69 K antigen serotypes in clinical diagnosis. In February 1996, a food poisoning event caused by a new O3:K6 serotype in India, the clone quickly spread world and became the pandemic clone.⁹ Group-specific PCR (GS-PCR) method can distinguish the O3:K6 strains isolated before and after 1996.⁹ The pandemic clone of *V. parahaemolyticus* had the following characteristics: *tdh*⁺, *trh*⁻, *toxRS/new*⁺ (a unique *toxRS* sequence detectable by GS-PCR) and *orf8*^{+/-} (the *orf8* sequence of f237 phage),^{9,10} which were found in at least 20 serotypes, including O3:K6, O4:K68, O4:K8, O1:K25 and O1:KUT (K untypeable).¹¹ The emergence of these pandemic characteristics in other serotype strains was understood to be the result of serotype transformation.

The virulence factors of *V. parahaemolyticus* include thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH), which can form 2 nm pores on the cell membrane, allowing water and ions to enter freely, thus making the red blood cells have osmotic dissolution and hemolytic activity.^{12,13} Type III secretion systems (T3SSs) are a needle like structure composed of 20–30 proteins. Their function is to secrete and inject virulence factors, cause apoptosis and produce cytotoxic effects.¹⁴ T3SS1 genes were present in all *V. parahaemolyticus* strains. T3SS2 α genes were present and co-regulate with *tdh* genes, whereas T3SS2 β genes were generally only found in *trh* strains.^{15–17}

Southeastern China, located on the west bank of the Pacific Ocean, is a place where there are frequent outbreaks of *V. parahaemolyticus*. We have been monitoring the pathogen spectrum of patients with acute diarrhoea in this area since 2009. *V. parahaemolyticus* ranked the second most prevalent bacterial pathogen, following diarrhoeagenic *Escherichia coli* (DEC).¹⁸ The fluctuation of serotype, genotype and drug sensitivity of *V. parahaemolyticus* were also observed. In this study, we analysed the pathogenic characteristics of and variation in *V. parahaemolyticus* isolated from acute diarrhoeal patients in southeastern China from 2013 to 2017 in an effort to discover the epidemic trend to prevent *V. parahaemolyticus* outbreaks in the future.

Materials and Methods

Ethics

The study protocol was approved by the Ethics Committees of the First Affiliated Hospital, College of Medicine, Zhejiang University and all participants provided written informed consent, including children, whose parent or guardian provided informed consent on their behalf. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Sample Collection and Microbiological Analysis

Stool specimens were collected from acute diarrhoeal patients (three or more watery or loose stools a day with a duration of no more than 14 days) in 6 general hospitals and 1 children's hospital in Zhejiang Province from 2013 to 2017. Clinical information regarding the patients was collected by filling out the information questionnaire of acute diarrhoeal cases. Stool specimens were cultured in thiosulfate citrate bile salt sucrose agar culture medium after alkaline peptone water (APW) enrichment to isolate microorganisms.¹⁹ *V. parahaemolyticus* was identified by MALDI Biotyper microbial identification system (Bruker, Germany) according to the instructions.

Serotyping

V. parahaemolyticus isolates were serotyped using agglutination tests with 11 O (lipopolysaccharide) and 65 K (capsule) antisera (Denka Seiken Ltd., Japan) according to the instructions provided with the reagents. Serotypes were defined as a unique combination of O and K. OUT was O untypeable and KUT was K untypeable.

Detection of Virulence-Associated Genes

The species-specific marker (*tlh*) and haemolysin genes (*tdh* and *trh*) were tested by real-time PCR.^{20,21} Pandemic markers (*toxRS/new* and *orf8*) were detected by monoplex PCR.¹⁰ The type III secretion system 1 (T3SS1) genes VP1670 (*vscP*), VP1686 (*copS*), VP1689 (*vscK*) and VP1694 (*vscF*), the T3SS2 α genes VP1362 (*vopB2*), VP1339 (*vscC2*), VP1335 (*vscS2*) and VP1327 (*vopT*), and the T3SS2 β genes (*vscC2*, *vopB2*, *vopC* and *vscS2*) were detected by four-multiplex PCR.²² The pathogenic group was defined as *tdh*⁺ or *trh*⁺ and the non-pathogenic group was *tdh*⁻*trh*⁻. The pandemic group was defined as *tdh*⁺, *trh*⁻, *toxRS/new*⁺ and *orf8*^{+/-} and all other isolates were assigned to the non-pandemic group.²³

Antimicrobial Susceptibility Testing

The Kirby-Bauer method was used for in vitro antimicrobial sensitivity testing using 18 conventional antibiotics.^{24,25} ampicillin, piperacillin/tazobactam, piperacillin, cefazolin, cefuroxime, ceftazidime, cefotaxime, cefepime, ceftoxitin, imipenem, meropenem, amikacin, gentamycin, ciprofloxacin, levofloxacin, trimethoprim/sulfamethoxazole, tetracycline, and chloramphenicol. The interpretation of susceptibility, mediation and drug resistance were determined according referred to the latest standard recommended by M45-Ed3E standards formulated by Clinical and Laboratory Standards Institute.²⁶

Statistical Analysis

Statistical analyses were performed using the statistical package for social sciences version 18 (SPSS Inc., USA). For statistical comparisons, the Chi-square test or Fisher's exact test was used for proportions, and t tests were used for means. All p-values were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Epidemiology

From 2013 to 2017 in southeastern China, 1220 (7.4%) *V. parahaemolyticus* strains were isolated from 16,504 stool specimens collected from acute diarrhoeal patients.

Table 1 Detection and Distribution of *Vibrio parahaemolyticus* in Patients with Acute Diarrhoea in Southeastern China from 2013 to 2017

Year	Samples	Positive	Rate % (No. of Positive/Samples)
2013	3348	204	6.1
2014	2892	205	7.1
2015	1874	142	7.6
2016	3735	266	7.1
2017	4655	403	8.7

The annual isolation rate fluctuated between 6.1% and 8.7%, and the results are shown in Table 1.

A total of 96.7% (1179/1220) of the *V. parahaemolyticus* isolates were isolated in summer and autumn, 69.6% in people aged 18–44, and there was no difference between males and females. The demographic characteristics of the patients with acute diarrhoea are listed in Table 2.

Serotypes

Among the 1220 *V. parahaemolyticus* isolates, fifty-nine serotypes were identified. The agglutination rate of the O antigen was 98.5% (1202/1220), of which O3 was the highest, accounting for 49.0% (598/1220). The agglutination rate of the K antigen was 59.0% (720/1220) and the

Table 2 Demographic Characteristics of the Patients with Acute Diarrhoea from Which *Vibrio parahaemolyticus* Was Isolated in Southeastern China from 2013 to 2017

Variable and Category	Total Cases	Vibrio Parahaemolyticus		P
		Cases (%)	Isolation Rate (%)	
Gender				0.982*
Male	7889	596(48.9)	7.6	
Female	7836	592(48.5)	7.6	
Unknown	789	32(2.6)	4.1	
Age				<0.001*
≤4	3953	12(1.0)	0.3	
5–17	938	64(5.2)	6.8	
18–44	6758	849(69.6)	12.6	
45–59	1919	176(14.4)	9.2	
≥60	2147	87(7.1)	4.1	
Unknown	789	32(2.6)	4.1	
Time				<0.001
March-May	2308	40(3.3)	1.7	
June-August	10,182	917(75.2)	9.0	
September-November	3450	262(21.5)	7.6	
December-February	564	1(0.1)	0.2	

Notes: *The P value does not include cases of unknown sex and age.

highest was K6, accounting for 45.7% (557/1220). O3:K6 was the most prevalent serotype, with 546 strains (44.8%), followed by O4:KUT (415 strains, 34.0%), O4:K8 (40 strains, 3.3%), O3:KUT (30 strains, 2.5%) and O10:K60 (25 strains, 2.0%). The serotype distribution of *V. parahaemolyticus* is shown in Table 3.

There were differences in serotype distribution between 2013 and 2017 (Table 3, Figure 1). From 2014 to 2016, the dominant serotype was O3:K6, accounting for 53.7%, 64.1% and 53.0% of the strains in those years, respectively. The O4:KUT serotype was predominant in 2013 and 2017, accounting for 43.6% and 40.9%

Table 3 Distribution of Serotypes of *Vibrio parahaemolyticus* Isolates from Patients with Acute Diarrhoea in Southeastern China from 2013 to 2017

O Serotypes (No. of Isolates*)	Serotype (No. of Isolates)				
	2013 (n=204)	2014 (n=205)	2015 (n=142)	2016 (n=266)	2017 (n=403)
O1(42)	O1:K36(4) O1:K56(1) O1:K63(1) O1:K9(1) O1:KUT†(8)	O1:K36(1) O1:KUT(1)	O1:K17(1) O1:K36(1) O1:K41(1) O1:KUT(2)	O1:K25(2) O1:K36(2) O1:K56(1) O1:KUT(5)	O1:K1(1) O1:K36(1) O1:K4(2) O1:K5(1) O1:K8(1) O1:KUT(4)
O2(9)	O2:K3(2)	–	O2:K3(1)	O2:K3(2)	O2:K28(3) O2:K3(1)
O3(598)	O3:K21(1) O3:K29(4) O3:K57(2) O3:K6(52) O3:KUT(3)	O3:K29(4) O3:K46(1) O3:K53(1) O3:K57(2) O3:K6(110) O3:KUT(1)	O3:K6(91) O3:K68(1) O3:KUT(1)	O3:K54(1) O3:K6(141) O3:KUT(4)	O3:K1(2) O3:K19(1) O3:K48(1) O3:K5(1) O3:K6(152) O3:KUT(21)
O4(491)	O4:K13(5) O4:K4(1) O4:K60(1) O4:K68(1) O4:K8(12) O4:K9(2) O4:KUT(89)	O4:K13(1) O4:K4(1) O4:K42(1) O4:K48(1) O4:K60(1) O4:K68(1) O4:K8(4) O4:K9(2) O4:KUT(56)	O4:K25(1) O4:K44(1) O4:K48(1) O4:K6(1) O4:K8(6) O4:KUT(25)	O4:K55(1) O4:K6(1) O4:K60(1) O4:K68(1) O4:K8(4) O4:KUT(80)	O4:K10(1) O4:K33(1) O4:K4(1) O4:K53(2) O4:K6(4) O4:K8(14) O4:KUT(165)
O5(14)	O5:K17(1) O5:KUT(2)	O5:K52(1) O5:KUT(3)	O5:K6(1) O5:K60(1)	O5:KUT(2)	O5:K17(1) O5:KUT(2)
O6(3)	–	O6:KUT(1)	O6:K18(1)	O6:K18(1)	–
O8(6)	O8:K41(2) O8:KUT(1)	O8:K41(1)	–	–	O8:K41(1) O8:KUT(1)
O10(32)	O10:K60(5)	O10:K60(5) O10:K8(1) O10:KUT(1)	O10:K60(2)	O10:K60(7) O10:KUT(2)	O10:K60(6) O10:K8(2) O10:KUT(1)
O11(7)	O11:KUT(2)	O11:KUT(2)	O11:KUT(2)	O11:KUT(1)	–
OUT† (18)	OUT:K59(1)	OUT:KUT(1)	OUT:KUT(1)	OUT:K6(3) OUT:KUT(4)	OUT:K38(1) OUT:K6(1) OUT:KUT(6)

Notes: *No. of isolates per serotype; †UT, untypeable; - indicates that this serotype was not detected in this year.

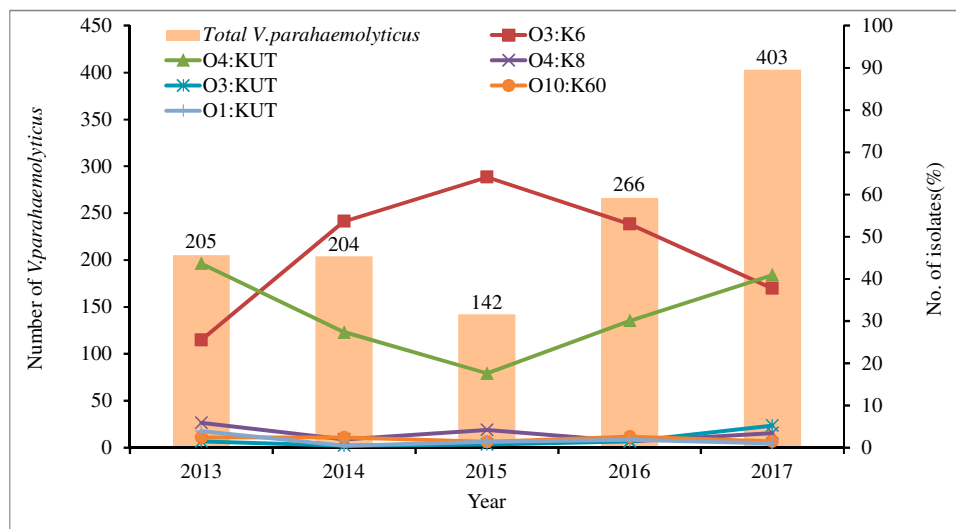


Figure 1 Distribution of the main *Vibrio parahaemolyticus* serotypes from patients with acute diarrhoea in southeastern China from 2013 to 2017.

respectively. The serotypes O3:K6, O4:KUT, O4:K8, O3:KUT, O10:K60, O1:KUT and O1:K36 appeared every year from 2013 to 2017. O4:K6 and O10:K60 serotypes began to appear after 2014 and 2015 respectively, while O3:K29, O4:K13, O3:K57 and O4:K9 serotypes have not been detected since 2013.

Distribution of Virulence-Associated Genes

A total of 1169 pathogenic strains (95.8%, 1169/1220) were detected, of which 1150 strains (94.3%) were tdh^+trh^- , 13 strains were tdh^+trh^+ and 6 strains were tdh^-trh^+ . Fifty-one strains (4.2%) did not carry pathogenic virulence genes (tdh^-trh^-). From 2013 to 2017, tdh^+trh^- pathogenic strains were dominant, accounting for more than 90%. There was no significant difference between different years ($P=0.878$), and the results are shown in Table 4. In 1169 pathogenic strains, there were 51 serotypes. In O3:K6 serotype isolates, 97.5% (14/557) were pathogenic strains, while the rate of pathogenic strains was

99.5% (413/415) for the O4:KUT serotype isolates. There were 27 serotypes of 51 non-pathogenic strains, of which O1:KUT and O5:KUT were the most abundant serotypes, with 5 strains (5.9%) each.

Among 1220 strains of *V. parahaemolyticus*, 49.5% (604/1220) were pandemic strains. The proportion of pandemic strains varied in different years ($P<0.05$), with pandemic strains predominating in 2015 and 2016, and non-pandemic strains predominating in 2013, 2014 and 2017 (Table 5). A total of 604 pandemic strains consisted of 26 serotypes, mainly O3:K6 (80.5%, 486/604), followed by O10:K60 (4.0%, 24/604) and O3:KUT (3.8%, 23/604), while 616 non-pandemic strains consisted of 51 serotypes, mainly O4:KUT (64.3%, 396/616), followed by O3:K6 (9.7%, 60/616) and O4:K8 (5.7%, 35/616), as shown in Figure 2. From 2013 to 2017, the proportions of O3:K6 in pandemic strains were 69.1%, 88.2%, 88.2%, 83.4% and 74.7%, respectively.

A total of 1128 strains (92.5%, 1128/1220) of *V. parahaemolyticus* carried the four T3SS1 genes.

Table 4 Haemolysin Genes of *Vibrio parahaemolyticus* Isolates from Patients with Acute Diarrhoea in Southeastern China from 2013 to 2017

Year	Total (n=1220)	Haemolysin Genes (%)			
		tdh^+trh^- (n= 1150)	tdh^+trh^+ (n= 13)	tdh^-trh^+ (n=6)	tdh^-trh^- (n=51)
2013	204	194(95.1)	1(0.5)	2(1.0)	7(3.4)
2014	205	192(93.7)	1(0.5)	2(1.0)	10(4.9)
2015	142	136(95.8)	3(2.1)	1(0.7)	2(1.4)
2016	266	250(94.0)	3(1.1)	0(0.0)	13(4.9)
2017	403	378(93.8)	5(1.2)	1(0.2)	19(4.7)

Notes: + positive, presence of the gene; - negative, absence of the gene.

Table 5 Distribution of Virulence Genes and Pandemic Markers in *Vibrio parahaemolyticus* Isolates from Patients with Acute Diarrhoea in Southeastern China from 2013 to 2017

Haemolysin Genes			Pandemic Markers		2013 (n=204)	2014 (n=205)	2015 (n=142)	2016 (n=266)	2017 (n=403)
tlh	tdh	trh	toxRS/ new	orf8					
Pandemic (49.5%, 604/1220)					68(33.3)	93(45.4)	85(59.9)	160(60.2)	198(49.1)
+	+	-	+	+	60(29.4)	70(34.1)	76(53.5)	131(49.2)	182(45.2)
+	+	-	+	-	8(3.9)	23(11.2)	9(6.3)	29(10.9)	16(4.0)
Non-pandemic (50.5%, 616/1220)					136(66.7)	112(54.6)	57(40.1)	106(39.8)	205(50.9)
+	+	-	-	-	124(60.8)	82(40.0)	41(28.9)	85(32.0)	177(43.9)
+	+	-	-	+	2(1.0)	17(8.3)	10(7.0)	5(1.9)	3(0.7)
+	+	+	+	+	1(0.5)	0(0.0)	0(0.0)	1(0.4)	1(0.2)
+	+	+	+	-	0(0.0)	0(0.0)	0(0.0)	1(0.4)	0(0.0)
+	+	+	-	-	0(0.0)	1(0.5)	3(2.1)	1(0.4)	4(1.0)
+	-	-	+	-	0(0.0)	0(0.0)	0(0.0)	4(1.5)	2(0.5)
+	-	-	-	-	7(3.4)	10(4.9)	1(0.7)	9(3.4)	17(4.2)
+	-	-	-	+	0(0.0)	0(0.0)	1(0.7)	0(0.0)	0(0.0)
+	-	+	-	-	2(1.0)	2(1.0)	1(0.7)	0(0.0)	1(0.2)

Among the *tdh*⁺ strains, 95.1% (1106/1163) carried the four T3SS2 α genes. All four T3SS2 α genes were negative in three strains. Ten of 19 *trh*⁺ strains carried the four T3SS2 β genes. No *tdh*⁻*trh*⁻ strain carried all four T3SS2 α or T3SS2 β genes. From 2013 to 2017 years, the positive rates of the four T3SS1 genes were 88.7%, 86.3%, 97.2%, 91.0% and 96.8% respectively ($p=0.000$). Among the *tdh*⁺ strains, 97.4%, 94.3%, 88.0%, 98.0% and 95.8% respectively carried the four T3SS2 α genes ($p=0.001$). The results of type III secretion system related genes are shown in Table 6.

Antimicrobial Profile

Most isolates were resistant to ampicillin, and the resistance rate fluctuated between 79.6% and 92.7% during 2013–2017. Nearly half of the isolates exhibited intermediate levels of susceptibility to cefuroxime (24.6–64.1%) and cefazolin (28.4–65.5%). More than 95% of isolates were sensitive to other antibacterial agents including fluoroquinolones, aminoglycosides, tetracyclines, trimethoprim-sulphamethoxazole, third-generation and fourth-generation cephalosporins, β -lactamase inhibitor combinations during 2013–2017. No isolate was resistance to carbapenems in five years. The antimicrobial susceptibilities of *V. parahaemolyticus* fluctuated with years, and the results are shown in Table 7.

Discussion

V. parahaemolyticus is a common pathogen that induces diarrhoea. In our study, the annual separation rate of *V. parahaemolyticus* in acute diarrhoeal patients was between 6.1% and 8.7%, which was close to the 8.1% isolation rate in this region between 2009 and 2013,²⁷ and the 6.0% isolation rate reported in southern China between 2007 and 2012.²⁸ However, the rate was higher compared with the 4.5% rate in Shanghai between 2012 and 2016,²⁹ and the 5.1% rate in northwestern Mexico between 2004 and 2010.³⁰ The difference may be related to geographical location and local eating habits, or even to sample selection and laboratory testing. In our study, 96.7% of the *V. parahaemolyticus* isolates were isolated between June and November, mainly in people aged 18–44. This result may be attributed to the optimum growth temperature of *V. parahaemolyticus* and the fact that young adults are more likely to eat in restaurants, thus increasing the risk of raw or inadequately cooked seafood. In early summer the temperature is 15°C or higher, and *V. parahaemolyticus* is released into the water column from the sediments where it survived during winter.³¹

Serotyping the 1220 *V. parahaemolyticus* isolates revealed that the agglutination rate of the O antigen was 98.5% and that of the K antigen was 59.0% which was much lower than any previously recorded rate.²⁷ O3:K6 was the main prevalent serotype, with 546 strains (44.8%),

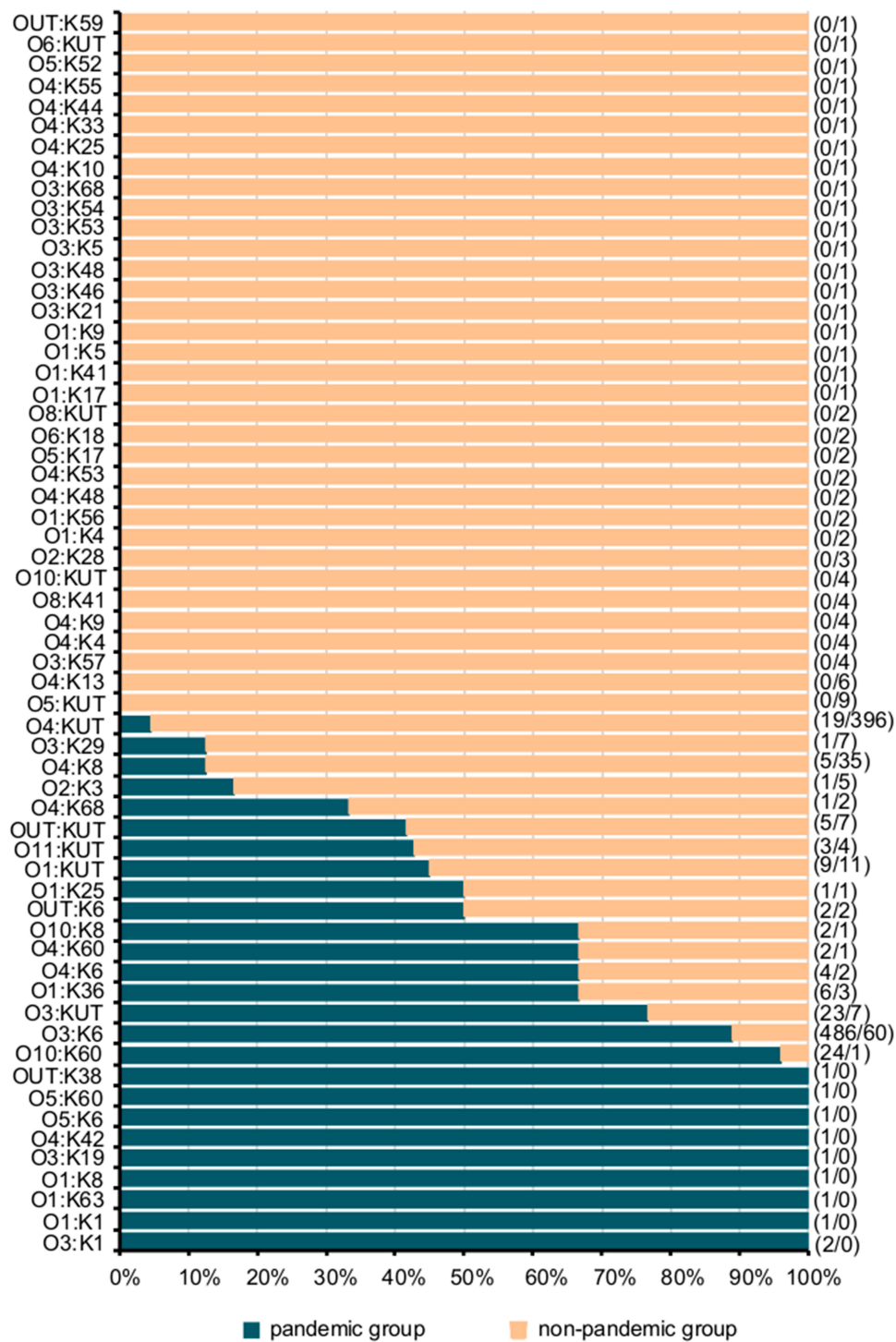


Figure 2 Distribution of different serotypes of *Vibrio parahaemolyticus* isolates in pandemic and non-pandemic strains. The number on the left side of the “/” in the parenthesis on the right side of the figure is the number of pandemic strains, and the number on the right side is the number of non-pandemic strains.

followed by O4:KUT (415/1220, 34.0%) and O4:K8 (40/1220, 3.3%). Since 1996, a new O3:K6 strain appeared in Kolkata, India.³² The O3:K6 strain rapidly spread throughout in the world in subsequent years and became a pandemic strain in clinical *V. parahaemolyticus* isolates.^{7,9,10,33,34} It is

worth noting that from 2013 to 2017 in southeastern China, the detection rate of the O3:K6 serotype was reduced and the second dominant serotype O4:KUT reached up to 34.0%. Even in 2013 and 2017, O4:KUT surpassed O3:K6 to become the dominant serotype, accounting for 43.6% and

Table 6 Distribution of T3SS Genes Among *Vibrio parahaemolyticus* Strains Isolated from Patients with Acute Diarrhoea in Southeastern China During 2013–2017

Genes	Total (n=1220)				2013 (n=204)				2014 (n=205)				2015 (n=142)				2016 (n=266)				2017 (n=403)			
	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
tdh	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
trh	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
n	1150	13	6	51	194	1	2	7	192	1	2	10	136	3	1	2	250	3	0	13	378	5	1	19
T3SS1																								
VP1670[<i>vscP</i>]	1144	13	6	51	192	1	2	7	191	1	2	10	136	3	1	2	248	3	0	13	377	5	1	19
VP1686[<i>vopS</i>]	1102	12	6	44	183	1	2	6	171	1	2	9	136	3	1	2	239	2	0	9	373	5	1	18
VP1689[<i>vscK</i>]	1129	13	6	48	189	1	2	6	179	1	2	10	136	3	1	2	248	3	0	12	377	5	1	18
VP1694[<i>vscF</i>]	1114	12	5	36	183	1	2	5	182	1	2	7	133	3	0	2	245	2	0	7	371	5	1	15
All four genes	1078	12	5	33	173	1	2	5	167	1	2	7	133	3	0	2	236	2	0	4	369	5	1	15
T3SS2α																								
VP1362[<i>vopB2</i>]	1120	4	2	0	190	1	1	0	182	0	0	0	125	0	0	0	247	2	0	0	376	1	1	0
VP1339[<i>vscC2</i>]	1137	4	2	0	192	1	1	0	192	0	0	0	135	0	0	0	249	2	0	0	369	1	1	0
VP1335[<i>vscS2</i>]	1143	4	2	0	192	1	1	0	191	0	0	0	136	0	0	0	249	2	0	0	375	1	1	0
VP1327[<i>vopT</i>]	1132	4	2	0	192	1	1	0	183	0	0	0	136	0	0	0	248	2	0	0	373	1	1	0
All four genes	1108	4	2	0	189	1	1	0	182	0	0	0	125	0	0	0	246	2	0	0	366	1	1	0
T3SS2β																								
<i>vscC2</i>	0	8	0	0	0	1	0	0	0	1	0	0	0	3	0	0	0	3	0	0	0	0	0	0
<i>vopB2</i>	0	13	1	2	0	1	0	0	0	1	0	0	0	3	0	0	0	3	0	0	0	5	1	2
<i>vopC</i>	0	11	0	1	0	1	0	0	0	1	0	0	0	3	0	0	0	3	0	0	0	3	0	1
<i>vscS2</i>	0	13	1	2	0	1	0	0	0	1	0	0	0	3	0	0	0	3	0	0	0	5	1	2
All four genes	0	8	0	0	0	1	0	0	0	1	0	0	0	3	0	0	0	3	0	0	0	0	0	0

40.9% respectively. In addition, O4:K6 and OUT:K6 serotypes began to appear after 2014 and 2015 respectively, while O3:K29, O4:K13, O3:K57 and O4:K9 serotypes have not been detected since 2013. These changes in the serotypes of *V. parahaemolyticus* may be related to environmental changes, such as rising water temperature and acidification of seawater.¹¹

Among the 1220 *V. parahaemolyticus* isolates, pathogenic strains (*tdh*⁺ or *trh*⁺) accounted for 95.8% and pandemic strains (*tdh*⁺, *trh*⁻, *toxRS/new*⁺) accounted for 49.5% of all strains. Non-pandemic strains were predominant in 2013, 2014 and 2017. This is a newly discovered phenomenon. The main reason was that 95.4% (396/415) of the isolates of the O4:KUT serotype which was the first dominant serotype in 2013 and 2017 did not have the *toxRS/new*⁺ characteristic of the pandemic strain. Although O4:KUT is not a pandemic serovariant, its clinical prevalence has increased and the clinical symptoms of patients infected with O4:KUT were similar to those infected with O3:K6. Whether the O4:KUT serotype strain has other pathogenic factors remains to be further studied.

T3SS is associated with the pathogenicity of *V. parahaemolyticus*. The T3SS1 genes are present in all *V. parahaemolyticus* isolates. The T3SS2 α genes co-

regulated with the *tdh* and T3SS2 β genes are closely related to the *trh* gene.^{15,17} The results of this study were consistent with previous results.²⁷ All 1220 *V. parahaemolyticus* isolates had at least one of the T3SS1 genes and no *tdh*⁻*trh*⁻ strain carrying T3SS2 α or T3SS2 β gene was detected.

In this study, *V. parahaemolyticus* isolates were usually generally to common antibiotics, except ampicillin. A total of 1079 isolates (88.5%) showed a resistance to ampicillin. Similar results were observed in isolates from seafood.³⁵ However, the resistance rates of amikacin and cefuroxime in this study in Zhejiang Province were 0.7% and 3.4%, which were inconsistent with the resistance rates of clinical *V. parahaemolyticus* isolates in Shanghai at 90.5% and 92.6%.^{36,37} The reason for this difference might be that the Shanghai samples came from only one hospital and clustered events might have been included, while our samples came from 7 hospitals in different regions and the results were more reliable. Although *V. parahaemolyticus* remained highly sensitive to fluoroquinolone (such as ciprofloxacin and levofloxacin), which were commonly used to treat infectious diarrhea in clinic, a trend of fluoroquinolone resistance had emerged. Changes in drug-resistance rate were also observed in other species in the *Vibrionaceae* family. According to surveillance findings in

Table 7 Antimicrobial Susceptibility Testing of 1220 *Vibrio Parahaemolyticus* Strains Isolated from Patients with Acute Diarrhoea During 2013–2017

Antimicrobial Agent	Total (n=1220)		2013 (n=204)		2014 (n=205)		2015 (n=142)		2016 (n=266)		2017 (n=403)		P*
	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	
Ampicillin (AMP)	1079(88.5)	31(2.5)	177(86.8)	5(2.5)	190(92.7)	5(2.4)	113(79.6)	10(7.0)	235(88.3)	7(2.6)	364(90.3)	4(1.0)	0.002
Piperacillin/tazobactam (TZP)	5(0.4)	1212(99.4)	0(0.0)	204(100.0)	0(0.0)	205(100.0)	0(0.0)	142(100.0)	0(0.0)	266(100.0)	5(1.2)	395(98.0)	0.096
Piperacillin (PRL)	26(2.1)	1144(93.8)	4(2.0)	188(92.2)	0(0.0)	194(94.6)	3(2.1)	127(89.4)	2(0.8)	259(97.4)	17(4.2)	376(93.3)	0.002
Cefazolin (KZ)	88(7.2)	639(52.4)	5(2.5)	147(72.1)	12(5.9)	77(37.6)	25(17.6)	26(18.3)	15(5.6)	116(43.6)	31(7.7)	273(67.7)	<0.001
Cefuroxime (CXM)	42(3.4)	542(44.5)	1(0.5)	145(71.1)	12(5.9)	66(32.2)	8(5.6)	41(28.9)	4(1.5)	125(47.0)	17(4.2)	165(40.9)	0.002
Cefazidime (CAZ)	12(1.0)	1202(98.5)	0(0.0)	203(99.5)	0(0.0)	202(98.5)	2(1.4)	139(97.9)	1(0.4)	265(99.6)	9(2.2)	393(97.5)	0.018
Cefotaxime (CTX)	18(1.5)	1145(93.8)	4(2.0)	195(95.6)	1(0.5)	183(89.3)	3(2.1)	131(92.3)	1(0.4)	259(97.4)	9(2.2)	377(93.5)	0.164
Cefepime (FEP)	11(0.9)	857(70.2)	0(0.0)	158(77.5)	0(0.0)	116(56.6)	0(0.0)	78(54.9)	1(0.4)	187(70.3)	10(2.5)	318(78.9)	0.004
Cefoxitin (FOX)	22(1.8)	1119(91.7)	4(2.0)	198(97.1)	1(0.5)	178(86.8)	2(1.4)	114(80.3)	1(0.4)	258(97.0)	14(3.5)	371(92.1)	0.021
Imipenem (IPM)	0(0.0)	1220(100.0)	0(0.0)	204(100.0)	0(0.0)	205(100.0)	0(0.0)	142(100.0)	0(0.0)	266(100.0)	0(0.0)	403(100.0)	-
Meropenem (MEM)	0(0.0)	1220(100.0)	0(0.0)	204(100.0)	0(0.0)	205(100.0)	0(0.0)	142(100.0)	0(0.0)	266(100.0)	0(0.0)	403(100.0)	-
Amikacin (AK)	9(0.7)	966(79.2)	1(0.5)	176(86.3)	1(0.5)	150(73.2)	1(0.7)	93(65.5)	1(0.4)	201(75.6)	5(1.2)	346(85.9)	0.815
Gentamycin (GN)	8(0.7)	1188(97.3)	0(0.0)	203(99.5)	0(0.0)	204(99.5)	0(0.0)	139(97.9)	1(0.4)	258(97.0)	7(1.7)	384(95.3)	0.054
Ciprofloxacin (CIP)	15(1.2)	1173(96.2)	0(0.0)	203(99.5)	0(0.0)	199(97.1)	3(2.1)	132(93.0)	2(0.8)	262(98.5)	10(2.5)	377(93.5)	0.013
Levofloxacin (LEV)	11(0.9)	1207(98.9)	0(0.0)	204(100.0)	0(0.0)	205(100.0)	2(1.4)	140(98.6)	1(0.4)	265(99.6)	8(2.0)	393(97.5)	0.035
Trimethoprim/ sulfamethoxazole (SXT)	35(2.9)	1179(96.6)	2(1.0)	202(99.0)	2(1.0)	203(99.0)	6(4.2)	136(95.8)	3(1.1)	262(98.5)	22(5.5)	376(93.3)	0.001
Tetracycline (TE)	37(3.0)	1177(96.5)	4(2.0)	200(98.0)	0(0.0)	204(99.5)	5(3.5)	135(95.1)	5(1.9)	261(98.1)	23(5.7)	377(93.5)	<0.001
Chloramphenicol (C)	14(1.1)	1195(98.0)	0(0.0)	203(99.5)	0(0.0)	205(100.0)	3(2.1)	135(95.1)	0(0.0)	265(99.6)	11(2.7)	387(96.0)	0.001

Note: P*, difference in resistance rates.

Nepal, resistance patterns in *Vibrio cholerae* were shown dramatic fluctuations in resistance to routinely used antibiotics. Resistance to ampicillin decreased from 93% in 2006 to 18% by 2010 and again raised to 100% by 2016. Ciprofloxacin and tetracycline resistance emerged in 2007, reached a peak during 2010–2012 and declined to 0 by 2016.³⁸ In addition, *Aeromonas spp.*, which is one of the main pathogens of infectious diarrhea, was uniformly resistant to penicillin, cephalosporins or carbapenems due to the production of various β -lactamases. From 2011 to 2017, *Aeromonas* demonstrated the high resistance rates to ceftriaxone (29.4%), ceftazidime (28.9%), cefepime (22.2%), ciprofloxacin (27.3%) and trimethoprim-sulfamethoxazole (45%), respectively, in southwest China.³⁹ In a 10-year retrospective survey in Hungary, the resistance trends of *Aeromonas* were showed.⁴⁰ The highest resistance levels overall was observed for ceftriaxone (20%), sulphamethoxazole/trimethoprim (18%), ciprofloxacin (14%) and cefepime (13.0%). Meropenem resistance was also above 10%, while resistance rates against doxycycline, tigecycline and gentamicin were around or lower than 5%. There was a significant increase in the number of resistant isolates corresponding to ceftriaxone, sulphamethoxazole/trimethoprim and meropenem. The variation in the antimicrobial resistance may be linked to how heavily antimicrobial drugs are used. In recent years, the consumption of antibiotics in aquaculture has increased globally to prevent or treat related diseases, leading to an increase in resistance rate of related bacteria, such as *V. parahaemolyticus* and *Aeromonas*. It is obvious that the pace of antibiotic drug discovery cannot keep up with the continuous and detrimental changes in resistance trends. Therefore, it is important to preserve the drugs that we currently have (through the development of rapid and sensitive diagnostic tools to ensure their prudent use, and antibiotic stewardship practices), in addition to facilitating the development of new ideal antibacterial drugs.⁴¹

Conclusions

In summary, *V. parahaemolyticus* was present at high levels in southeastern China from 2013 to 2017. Although the pandemic O3:K6 serotype was predominant, new serotypes continue to emerge, especially the O4:KUT serotype, which exceeded O3:K6 in prevalence in some years. Fluctuations in the virulence gene distribution and drug sensitivity of these isolates were obvious. Long-term surveillance is necessary to prevent the outbreak or transmission of this pathogen.

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Disclosure

The authors declare no competing interests in this study.

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