Infection and Drug Resistance

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ORIGINAL RESEARCH

Antibiotic Resistance and Epidemiology of *Vibrio parahaemolyticus* from Clinical Samples in Nantong, China, 2018–2021

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Purpose: The objective of this study was to investigate the prevalence and molecular characteristics of *Vibrio parahaemolyticus* isolates from fecal samples of patients in Nantong, China.

Methods: From 2018 to 2021, a total of 106 clinical cases and samples of *V. parahaemolyticus* infection were collected. The virulence genes, serotypes and antibiotic resistance of these isolates were analyzed. Additionally, pulsed-field gel electrophoresis (PFGE) was used to analyze the homogeneity of the isolates.

Results: Outbreaks of *V. parahaemolyticus* infection were concentrated in the summer, with seafood consumption being the primary contributing factor, followed by meat and meat products. *tlh+tdh+trh-* was confirmed as the most frequently detected virulence genotype among the clinical isolates. 16 serotypes were identified, and O3:K6 was the dominant serotype in Nantong. The antimicrobial susceptibility testing revealed the highest resistance rate to cefazolin (99.1%, 104/106), followed by ampicillin (64.2%, 68/106) and tetracycline (29.2%, 31/106). Fourteen resistant phenotypes were identified, with ampicillin-cefazolin being the most prevalent. The multiple antibiotic resistance (MAR) index ranged from 0.07 to 0.36. PFGE typing clustered isolates with similarity greater than 85% into ten genetic clusters (A-J).

Conclusion: Clinical isolates generally exhibited pathogenicity and drug resistance, with some isolates displaying high homology. Clusters C, E, and G were the predominant circulating clusters in this area, posing a potential risk of recurrent outbreaks, which demanded our vigilance.

Keywords: *Vibrio parahaemolyticus*, clinical isolates, antibiotic resistance, PFGE

Introduction

Vibrio parahaemolyticus, a gram-negative halophilic bacterium, naturally exists in freshwater, estuarine, and marine environments.[1] Gastroenteritis caused by foodborne poisoning is the most common clinical symptom associated with *V. parahaemolyticus* infection, often linked to the consumption of raw or undercooked contaminated seafood.[2,3] In the United States, *V. parahaemolyticus* is considered a major cause of human gastroenteritis related to seafood consumption.[4] Meanwhile, coastal countries in Asia, such as China, Japan, and South Korea, also suffer from this bacterium.[5–7] A previous research report from Zhejiang Province in China indicated that *V. parahaemolyticus* had become the primary cause of local foodborne diseases, accounting for 58.4% of bacterial outbreaks.[8] Global climate change and increased trade had elevated the risk of *V. parahaemolyticus* infection, leading to greater attention on this bacterium.[9,10]

Virulence genes play a crucial role in the pathogenicity of *V. parahaemolyticus*. Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are considered key virulence factors of this bacterium, encoded by the *tdh* and *trh* genes, respectively.[11] *tdh* and *trh* exhibit similar biological properties, including hemolytic activity, enterotoxicity, and cytotoxicity, with a sequence homology of 70%.[12] Therefore, these genes are used as molecular markers

Received: 18 August 2023
Accepted: 29 November 2023
Published: 4 December 2023

for the pathogenic capacity of *V. parahaemolyticus*. Epidemiological investigations found high detection rates of these virulence genes in clinical isolates, while they were rarely detected in environmental isolates.[13,14] *tlh* is considered a specific marker for *V. parahaemolyticus* and is frequently used for species identification; however, its pathogenicity has not been elucidated.[15] Reports on antibiotic resistance in Vibrio species had been increasingly frequent, with multiple antimicrobial agents gradually losing effectiveness.[16–18] Resistance of *V. parahaemolyticus* strains to ampicillin had increased worldwide,[6] and many isolates had developed multidrug resistance.[17,19] Multiple antibiotic resistance in Vibrio species had been a major global public health concern, contributing to increased patient mortality rates.[20]

Serotyping is a common method for typing *V. parahaemolyticus*.[21] Through accurate serotyping, the serotypes prevalent in different regions can be determined. The research on V. parahaemolyticus became highly significant following the appearance of the O3:K6 clone in Kolkata, India, in 1996, subsequently spreading globally and causing the pandemic. Later, variants of this strain, including O4:K68, O1:K25, and O1:KUT, were recognized for exhibiting a dissemination pattern resembling that of O3:K6.[22] The emergence of new serotypes and potential ambiguities during the serotyping process pose challenges in foodborne epidemiological investigations and traceability. Over the past few decades, a multitude of molecular typing techniques related to foodborne pathogens has emerged, including pulsed-field gel electrophoresis (PFGE), repetitive extragenic palindromic PCR (REP-PCR), multilocus sequence typing (MLST), multilocus variable-number tandem repeat analysis (MLVA), and clustered regularly interspaced short palindromic repeats (CRISPR). These techniques have proven to be valuable tools for providing genetic relatedness information.[23] Each of the mentioned techniques has its own advantages and disadvantages in terms of speed, sensitivity, resolution, and implementation cost.[17] PFGE had previously been demonstrated as a suitable method for *V. parahaemolyticus* typing and was considered the "gold standard" for molecular typing of foodborne pathogens.[24,25]

Nantong is located in the eastern coastal region of China, known for its abundant marine resources. The local residents have unique dietary habits and frequently consume a variety of seafood, such as clams, drunken shrimp (live shrimp marinated in alcohol), and marine fish, which are often associated with cases of foodborne illnesses related to *V. parahaemolyticus*. The southeastern coastal region of China is considered a hotspot for *V. parahaemolyticus prevalence*.[26] However, there are limited research reports regarding the epidemiology of this bacteria in Nantong. Therefore, we conducted a study on the antibiotic resistance, and epidemiological characteristics of *V. parahaemolyticus* strains isolated from clinical samples in this region. This research aims to provide insights for the assessment of infection risks and treatment by the health and prevention departments.

Materials and Methods

Collection of Samples and Sample Information

During the period of foodborne disease risk monitoring in multiple regions of Nantong City from 2018 to 2021, a total of 106 *V. parahaemolyticus* isolates were obtained from patient fecal samples, all collected from three sentinel hospitals in Nantong. By reviewing case reports and conducting remote telephone consultations with the infected individuals, we gained in-depth insights into the etiology of *V. parahaemolyticus* infections.

Isolation and Confirmation of *V. parahaemolyticus*

According to the Chinese National Standard for Food Safety Manual (GB 4789.7–2013), with slight modifications, isolation and identification of *V. parahaemolyticus* strains were performed. In brief, 1g of each sample was thoroughly mixed with 10mL of sterile 3% NaCl alkaline peptone water (3% NaCl APW; Huan Kai Microbial, China) and incubated at 37°C for 18 hours. Subsequently, a loopful of the bacterial suspension was streaked onto Vibrio chromogenic agar plates (Huan Kai Microbial, China) and incubated at 37°C for 24 hours. Single colonies exhibiting a diameter of 2–3 mm and displaying purple or fuchsia coloration were selected and confirmed using an automated microbial mass spectrometry detection system AUTOF MS1000 (Autobio, China).[27]

Virulence-Associated Genes in *V. parahaemolyticus* Isolates

A volume of 1mL of the isolated *V. parahaemolyticus* bacterial suspension was heated at 100°C for 10 minutes. Subsequently, the sample was centrifuged at 8000g for 5 minutes, and 200μL of the supernatant containing DNA was collected and stored at −80°C for later use. The presence of virulence-related genes in the 106 clinical isolates was determined using a commercial detection kit for *V. parahaemolyticus* (Chifeng Zhongkang Biotechnology, China). The reaction system was prepared according to the manufacturer's instructions, and the corresponding program for DNA amplification was set on an ABI 7500 fluorescence quantitative PCR instrument (Thermo Fisher Scientific, USA). The primer sequences of the three virulence genes were provided by Chifeng Zhongkang Biotechnology Co., Ltd., Inner Mongolia, China. (*tlh*-*F*: ATTAGATTTGGCGAACGA, *tlh*-R: ATTGCTGCGTCGTTGCTC; *tdh*-F: GCAGCGG TGTCTGGCTATAA, *tdh*-R: ACCTTCATCTTCACCAACAAAGT; *trh*-F: TTCAACGGTCTTCACAAA; *trh*-R: CGTTTCATCCAAATACGTTACACTT).

Serotyping

Serological analysis was performed using the *V. parahaemolyticus* serodiagnosis kit (Denka Seiken, Japan), which included 11 O (lipopolysaccharide) and 65 K (capsular) antisera. Individual colonies of isolates were streaked onto 3% sodium chloride tryptone soy agar plates (Huan Kai Microbial, China) and cultured at 37°C for 18 hours. Colonies were washed from the agar plates using a solution of 3% NaCl and 5% glycerol. A portion of the bacterial suspension was directly mixed with K-antigen antisera for slide agglutination reactions. Another portion of the bacterial suspension was subjected to autoclave at 121°C for 1.5 hours, followed by centrifugation at 12,000g for 15 minutes, and the supernatant was discarded. The pellet was washed three times with normal saline, and then centrifuged at 12,000g for 15 minutes. The final suspension was mixed with O-antigen antisera for agglutination reactions, with normal saline used as negative control.

Antimicrobial Susceptibility Testing

The sensitivity of 106 *V. parahaemolyticus* isolates to 14 antibiotics was tested using the broth microdilution minimum inhibitory concentration (MIC) method. A bacterial colony suspension was prepared in sterile 0.9% NaCl solution and adjusted to a 0.5 McFarland standard. The suspension was then inoculated onto the Gram-negative bacterial identification plates (BD, USA) and incubated for 24 hours in the fully automated bacterial identification/antibiotic susceptibility testing system BD Phoenix M-50 to obtain results. The testing concentrations for the 14 antibiotics on the sensitivity plates were as follows: ampicillin (2–32 μg/mL), ampicillin/sulbactam (1/0.5–32/16 μg/mL), cefotaxime (0.25–16 μg/mL), cefazolin (0.5–16 μg/mL), ceftazidime (0.5–16 μg /mL), cefoxitin (2–64 μg/mL), tetracycline (1–16 μg/mL), ciprofloxacin (0.015–2 μg/mL), nalidixic acid (4–32 μg/mL), gentamicin (0.5–16 μg/mL), trimethoprim/sulfamethoxazole (0.5/9.5–8/152 μg/mL), azithromycin (2–64 μg/mL), imipenem (0.25–8 μg/mL), chloramphenicol (4–32 μg/mL). Escherichia coli ATCC 25922 was used as the quality control (QC) strain. According to the antibiotic MIC breakpoints interpretation in the Clinical and Laboratory Standards Institute (CLSI, 2016) document M45, the test results were classified as susceptible (S), intermediate (I), and resistant (R). The detail interpretation criteria for MIC breakpoints are listed in Table S1.

The Multiple Antibiotic Resistance (MAR) index of the isolates was calculated using the method described by Krumperman.[28] The MAR index is defined as a/b, where a is the number of antibiotics to which a single isolate exhibits resistance, and b is the total number of antibiotics tested against that isolate. For example, the isolate NT2020127 was tested against 14 antibiotics and was resistant to 5 antibiotics, so its MDR index is 5/14. MAR index greater than 0.2 indicates a high level of antibiotic contamination in the environment, posing a potential risk to human health.[29]

PFGE Testing

According to the PulseNet International guidelines, PFGE testing was conducted using chromosomal DNA of the bacterial isolates digested with the restriction enzyme *Not* I (Takara, Japan). The digested gel blocks were loaded onto a 1% agarose gel and subjected to PFGE testing using the Chef Mapper pulsed-field electrophoresis system (Bio-Rad,

USA) in 0.5X Tris-borate-EDTA (TBE) buffer. The electrophoresis conditions were set as follows: 18 hours of electrophoresis time, initial switch time of 2.5 seconds, and final switch time of 60 seconds. After staining the gel with GleRed (Biotium, USA) for 20 minutes, the bands were observed, and BioNumerics version 7.6 software (Applied-Maths, Belgium) was used to analyze all bands and determine the PFGE patterns. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) and the Dice similarity coefficient with a position tolerance of 1.5%. Fingerprints with a similarity greater than 85% were grouped into the same gene cluster.[30] Salmonella strain H9812 (digested with *Xba*I) was used as the reference marker.

Statistical Analysis

The data was processed and analyzed using Excel 2016 (Microsoft, USA) and SPSS software 22.0 (SPSS Inc., USA). Descriptive statistics were employed to the drug sensitivity distribution and prevalence of clinical isolates of *V. parahaemolyticus* in Nantong. Graphs were generated using GraphPad Prism 8.0 (GraphPad Software Inc., USA).

Results

Epidemiological Investigation and Virulence Genotypes of *V. parahaemolyticus*

From January 2018 to December 2021, a total of 106 cases of *V. parahaemolyticus* infection were collected. Among these cases, 56.7% (60/106) were attributed to seafood consumption, while 25.4% (27/106) of the patients reported consuming meat and meat products. Additionally, a small portion of the patients (17.9%, 19/106) reported consuming other types of food. Table 1 provides a detailed breakdown of *V. parahaemolyticus* infections in patients caused by various types of food. Through the detection of virulence genes, it was found that the clinical isolates mainly exhibited the *tlh*+*tdh*+*trh*-phenotype (81.1%, 86/106). Some isolates presented as *tlh*+*tdh*-*trh*-(14.2%, 15/106), and only five isolates showed the *tlh* +*tdh*+*trh*+ gene combination (Table 1). Figure 1 illustrates the distinct seasonal patterns of *V. parahaemolyticus* outbreaks, with a higher incidence observed during the summer months and a peak in August (22.6%, 24/106). Conversely,

Table 1 Virulence Genotypes Presented by Clinical Isolates

Cases Caused by Different Food Types		No. of Isolates Carrying Different Virulence Genotypes			
		tlh+*tdh*-*trh*-	*tlh*+*tdh*+*trh*-	*tlh*+*tdh*+*trh*+	Total (%)
Seafood induced	Mixed seafood	2	18	1	21(19.8%)
	Clam	1	6	1	8(7.6%)
	Shrimp	2	4	0	6(5.6%)
	Blue crab	1	5	0	6(5.6%)
	Salmon	0	5	1	6(5.6%)
	Oysters	1	4	0	5(4.7%)
	Portunid	0	3	1	4(3.8%)
	Flounder	1	3	0	4(3.8%)
Meat and meat products induced	Pork	1	10	1	12(11.3%)
	Beef	2	6	0	8(7.6%)
	Duck	1	3	0	4(3.8%)
	Mixed meat	1	2	0	3(2.8%)
Other food induced	Eggs and egg products	0	4	0	4(3.8%)
	Unknown food	2	13	0	15(14.2%)
Total (%)		15(14.2%)	86(81.1%)	5(4.7%)	106(100%)

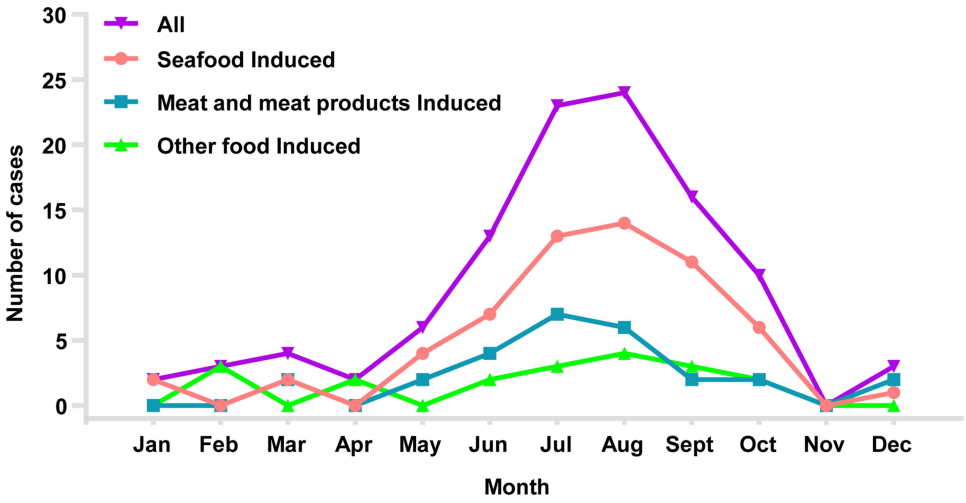

Figure 1 Monthly variation in *V. parahaemolyticus* cases associated with different food types in Nantong.

fewer cases were reported during the winter season. The consumption of seafood was identified as the primary contributing factor to the outbreaks during the summer, followed by meat and meat products.

Serotyping

A total of 16 serotypes were identified among the 106 *V. parahaemolyticus* isolates, with O3:K6 being the dominant type, accounting for 53.8% (57/106) of the isolates (Table 2). Among these, 86 isolates were fully typed, resulting in a complete typing rate of 81.1%. Additionally, 20 isolates had untyped K antigens, including O1:KUT (4.7%, 5/106), O3:KUT (4.7%, 5/106), O4:KUT (2.8%, 3/106), O7:KUT (1.9%, 2/106), O8:KUT (1.9%, 2/106), and O10:KUT (2.8%, 3/106).

Antibiotic Resistance of Isolates

In our study, antimicrobial susceptibility testing was conducted on 106 isolates of *V. parahaemolyticus* using nine antibiotic groups (Figure 2): penicillin and β-lactam/β-lactamase inhibitor, cephalosporins, tetracyclines, quinolones,

Table 2 Serotypes of 106 *V. parahaemolyticus* Isolates

Serotype	No. of Isolates	The Percentage (%)
O1:KUT	5	4.7
O1:K6	4	3.8
O1:K25	2	1.9
O2:K3	3	2.8
O3:KUT	5	4.7
O3:K6	57	53.8
O3:K8	4	3.8
O4:K8	5	4.7
O4:K10	4	3.8
O4:K12	1	0.9
O4:K68	2	1.9

(*Continued*)

Table 2 (Continued).

Serotype	No. of Isolates	The Percentage (%)
O4:KUT	3	2.8
O7:KUT	2	1.9
O8:KUT	2	1.9
O10:K4	4	3.8
O10:KUT	3	2.8
Total	106	100

Note: KUT stands for untyped K antigen.

phenicols, aminoglycosides, sulfonamides, macrolides, and carbapenems. All isolates exhibited sensitivity to ceftazidime and ciprofloxacin. The majority of isolates were sensitive to cefotaxime (98.1%, 104/106), ampicillin/sulbactam (96.2%, 102/106), gentamicin (94.3%, 100/106), nalidixic acid (93.4%, 99/106), cefoxitin (90.6%, 96/106), imipenem (87.7%, 93/106) and trimethoprim/sulfamethoxazole (75.5%, 80/106). Approximately 34.0% (36/106) and 25.5% (27/106) of the isolates showed intermediate susceptibility to azithromycin and chloramphenicol, respectively. However, 99.1% (105/106) of the isolates demonstrated resistance to cefazolin, and resistance was also observed against other drugs such as ampicillin (64.2%, 68/106) and tetracycline (29.2%, 31/106).

Table 3 documents fourteen antimicrobial resistance phenotypes of *V. parahaemolyticus*, with all isolates showing resistance to at least one antibiotic. The MAR index ranged from 0.07 to 0.36. The most common resistant phenotype was AMP-CFZ, observed in 24.5% (26/106) of the isolates, with a corresponding MAR index of 0.14. Among the isolates, 38.7% (41/106) exhibited resistance to three or more antibiotics, with MAR index values exceeding 0.20.

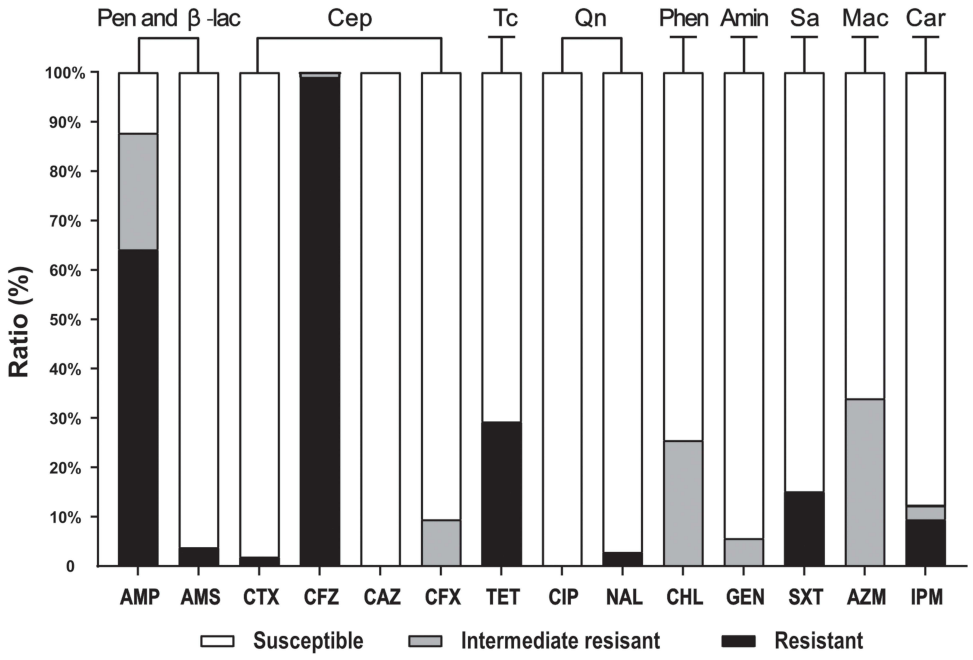

Figure 2 The percentage of antibiotic resistant of *V. parahaemolyticus* isolated from clinical samples.
Abbreviations: AMP, ampicillin; AMS, ampicillin/sulbactam; CTX, cefotaxime; CFZ, cefazolin; CAZ, ceftazidime; CFX, cefoxitin; TET, tetracycline; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; AZM, azithromycin; IPM, imipenem; Pen and β-lac, penicillin and β-lactam/β-lactamase inhibitor; Cep, cephalosporins; Tc, tetracyclines; Qn, quinolones; Phen, phenicols; Amin, aminoglycosides; Sa, sulfonamides; Mac, macrolides; Car, carbapenems.

Table 3 Resistance Phenotypes of *V. parahaemolyticus* Among 106 Resistant Isolates

Resistant Phenotypes	No. (%) of Isolates	No. of Antibiotics	MAR Index
AMP	1(0.9)	1	0.07
CFZ	22(20.8)	1	0.07
AMP-CFZ	26(24.5)	2	0.14
CFZ-TET	10(9.4)	2	0.14
CFZ-SXT	6(5.7)	2	0.14
AMP-AMS-CFZ	4(3.8)	3	0.21
AMP-CFZ-TET	13(12.3)	3	0.21
AMP-CFZ-SXT	5(4.7)	3	0.21
AMP-CFZ-CTX	2(1.9)	3	0.21
AMP-CFZ-NAL	3(2.8)	3	0.21
AMP-CFZ-IPM	6(5.7)	3	0.21
AMP-CFZ-TET-SXT	4(3.8)	4	0.29
AMP-CFZ-TET-IPM	3(2.8)	4	0.29
AMP-CFZ-TET-SXT-IPM	1(0.9)	5	0.36

Abbreviations: AMP, ampicillin; AMS, ampicillin/sulbactam; CTX, cefotaxime; CFZ, cefazolin; CAZ, ceftazidime; CFX, cefoxitin; TET, tetracycline; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; AZM, azithromycin; IPM, imipenem; MAR index, Multiple Antibiotic Resistance index.

PFGE Analysis

Following *Not* I enzyme digestion, 86 distinguishable patterns were generated among the 106 isolates, with similarity ranging from 47.1% to 100.0%. Using an 85% similarity cutoff value, the 77 isolates were divided into ten genetic clusters (A-J), while the remaining 29 isolates exhibited dispersed patterns, reflecting the genetic relatedness and diversity of *V. parahaemolyticus* in the studied region. Cluster C emerged as the dominant genetic cluster, comprising 26 isolates. Additionally, in descending order, the clusters were ranked as follows: G (15), E (7) = J (7), H (6), I (5), D (4), A (3), B (2) = F (2). Clusters C, E, and G contained isolates from different years. We observed that isolates within different genetic clusters could exhibit the same resistance phenotypes, while within the same cluster, isolates could represent either identical or different resistance phenotypes. Overall, no apparent correlation was observed between the resistance profiles, virulence genotypes, and genetic profiles (PFGE patterns) of the clinical isolates (Figure 3).

Discussion

From 2018 to 2021, we collected 106 isolates of *V. parahaemolyticus* from clinical samples in three hospitals in Nantong, China, analyzing their antibiotic resistance and epidemiological characteristics. Reports of *V. parahaemolyticus* infection cases were predominantly observed during the summer, showing significant differences compared to the winter season. This finding may be related to the variation in average temperatures between these two seasons,[31] which has also been observed in Huzhou, China.[24] The incidence of *V. parahaemolyticus* infection is increasing and often follows regional climate trends, particularly during unusually warm weather outbreaks.[9] Therefore, the prevention and control of this bacterium are particularly crucial during the summer and autumn seasons, especially in August.[8] Consumption of seafood poses the greatest risk for *V. parahaemolyticus* infection, and the role of meat and meat products as transmission vehicles should not be overlooked during outbreaks. Stains on meat are more viscous than on other contaminated foods, so they may be harder to remove, essentially retaining a lot of bacteria on the contact surface.[32] In a monitoring report on ready-

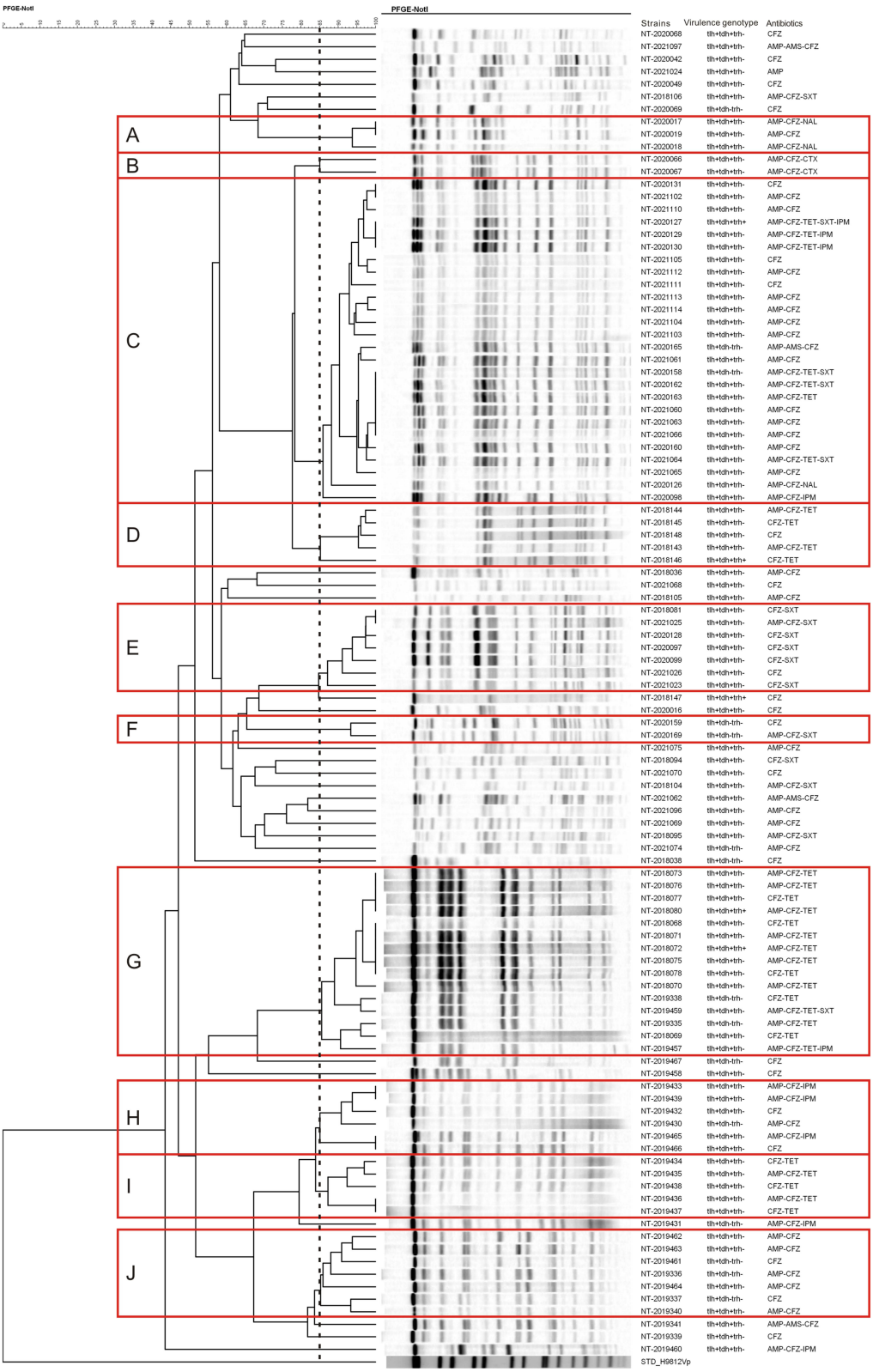

Figure 3 PFGE fingerprints, strain numbers, virulence genotypes and antibiotic resistance phenotypes of 106 clinical isolates. The red boxes represent different genetic clusters. The dashed lines represent a cutoff of 85% similarity.

Note: The first four digits of the strain number represent the year of isolation.

to-eat foods in China, 22 out of 371 samples of cooked meat tested positive for *V. parahaemolyticus* (5.9%). The positive isolates did not contain the *trh* and *tdh* genes.[33] According to literature reviews, research conducted in Hong Kong, Vietnam, and Indonesia found that the prevalence of *tlh* and *tdh* genes in seafood isolates is less than 10%.[34] However, in studies from South Korea, Malaysia, Mexico, and Germany, these genes were detected in over 50% of seafood isolates.[35–38] As shown in Table 1, 87 cases were associated with 8 different types of seafood and 4 different types of meat and meat products, with 87.3% (76/87) of isolates carrying the *tdh* or *trh* genes. This emphasizes the need for researchers to conduct monitoring of Vibrio parahaemolyticus in seafood, meat, and meat products in the region. In our report, all 106 isolates were positive for the *tlh* gene, consistent with the results from mass spectrometry confirming *V. parahaemolyticus* identification. Among the isolates, 85.9% (91/106) were positive for *tdh*, with 5 isolates also exhibiting *trh* positivity. The detection rate of pathogenic *V. parahaemolyticus* was similar in clinical samples from the southeast coast of China,[26] and higher than in the environment and sea food.[39] 14.2% (15/106) of the patients' stool samples tested positive for *V. parahaemolyticus*, with the absence of both *tdh* and *trh* virulence genes. However, they still exhibited severe diarrhea symptoms. This phenomenon has been reported in previous studies.[40]

To date, *V. parahaemolyticus* has been classified into 13 O serogroups and over 70 K serogroups based on the differentiation of its somatic O antigens and capsular K antigens.[41] However, isolates of *V. parahaemolyticus* with untypeable or new O or K antigens are frequently isolated.[42] The serotype distribution in Nantong City from 2018 to 2021 was similar to that in other regions of Jiangsu Province from 2006 to 2014.[23] O3:K6 was the dominant serotype, and no other dominant serotypes were identified. *V. parahaemolyticus* serotype O4:K12 caused gastroenteritis outbreaks in neighboring Shanghai in 2006, 2010, 2011, and 2014.[43] In this study, only one isolate with the O4:K12 serotype was found, indicating possible regional differences or changes in serotype over time. *V. parahaemolyticus* frequently undergoes recombination, and recombination in the vicinity of O- and K- antigen coding gene clusters contributes to the serotype transformation of the bacterium.[44]

The results of the antimicrobial susceptibility testing revealed a high level of resistance (99.1%, 105/106) of the clinical isolates of *V. parahaemolyticus* to cefazolin. This is similar to the recent findings of a resistance rate of 99.2% in seafood isolates from Nanjing, but significantly higher than the resistance rate (50.4%) of domestic clinical isolates ten years ago.[17,26] The misuse of first-generation cephalosporins may have occurred in recent years.[45] According to previous literature reports, *V. parahaemolyticus* isolates from the Maryland coastal bay in the United States showed no resistance to ceftazidime,[46] while isolates from fresh shrimps in Hong Kong exhibited low resistance (6%) to cefotaxime.[47] In comparison, Letchumanan et al reported that 73% of isolates from shellfish in Malaysia were resistant to cefotaxime, and 52% were resistant to ceftazidime.[48] In environmental and clinical isolates from the northwest Pacific coast of Mexico, approximately 20% were resistant to cefotaxime.[49] In this study, isolates exhibited low resistance to third-generation cephalosporins. All isolates were susceptible to ceftazidime, with only 1.9% (2/106) of isolates showing resistance to cefotaxime. Our data showed that 64.2% (68/106) of the isolates exhibited resistance to ampicillin, which aligns with the reported proportions of ampicillin-resistant *V. parahaemolyticus* ranging from 40% to 100% in different regions worldwide.[50] For instance, a study in Jordan reported that 43% of imported fish isolates demonstrated resistance to this antibiotic.[39] Similarly, Vu et al reported a resistance rate of 81.4% among seafood isolates in Vietnam.[51] Additionally, high rates of ampicillin resistance have been observed in clinical isolates from two different regions in China, reaching 86.8% and 98.5%, respectively.[24,52] These findings suggest that ampicillin may be ineffective for the treatment of *V. parahaemolyticus* infections.[45] Currently, tetracycline, cephalothin, and quinolone drugs are considered first-line options for the treatment of *V. parahaemolyticus* infections.[50,53,54] Unlike the study conducted by Su et al in Nantong,[52] we established resistance to tetracycline. It is worth noting that the resistance to tetracycline observed in 29.2% (31/106) of clinical isolates is higher than the average resistance (<10%) reported in global literature for marine, environmental, and clinical isolates.[39] Overall, considering the antimicrobial testing results, it is recommended to combine the use of broad-spectrum antibiotics with lower resistance, such as cephalosporins and quinolones, in clinical treatments in this region.

Due to its effectiveness and cost-efficiency, the MAR indexing has been widely recognized as an effective method for tracing bacterial sources. Therefore, the MAR index serves as a useful indicator for assessing the contamination risks that pose potential threats to human health.[55] In this study, over half of the isolates (61.3%, 65/106) exhibited MAR index

below 0.2, indicating a relatively low risk of antibiotic contamination in clinical settings in the region. However, the increasing trend of multidrug resistance in *V. parahaemolyticus* still warrants our attention. We identified an isolate (NT-2020127) that showed resistance to five antibiotics, with a MAR index of 0.36, demonstrating resistance to AMP-CFZ-TET-SXT-IPM. Other studies reported MAR index exceeding 0.2 in *V. parahaemolyticus* isolated from seafood; however, there were relatively fewer reports incorporating the MAR indexing into clinical isolates.[56,57] Differences in MAR index can be attributed to variances in sample origins, geographical distribution, and testing methods.[58]

The PFGE fingerprint patterns revealed a high degree of homogeneity among clinical isolates within the same genetic cluster. Approximately 73.6% (78/106) of the isolates exhibited clustering, which was associated with an increased occurrence of community-wide foodborne poisoning events related to *V. parahaemolyticus* in recent years. In this study, the isolates were predominantly concentrated in clusters C, E, and G, accounting for 48 isolates (45.3%, 48/106). Remarkably, these clusters comprised isolates detected in different years in the region, indicating a potential for future outbreaks. To prevent the recurrence of outbreaks, it is essential for relevant authorities to strengthen supervision and regulation in key areas such as restaurants, markets, and seafood farms. Li et al discovered different virulence factors in *V. parahaemolyticus isolates* with the same sequence type (ST) using MLST analysis.[23] This phenomenon was also observed in our research, where isolates within the same gene cluster exhibited diverse virulence genotypes. Combining antibiotic resistance phenotypes and PFGE molecular typing, we observed that isolates belonging to the same clusters exhibited diverse resistance phenotypes, characterized by the addition of new resistance phenotypes to existing ones. For instance, cluster A (AMP-CFZ, AMP-CFZ-NAL), cluster D (CFZ, CFZ-TET, AMP-CFZ-TET), cluster E (CFZ, CFZ-SXT, AMP-CFZ-SXT), and so on. This diversity in resistance patterns may be attributed to variations in antibiotic usage in different local seafood farming practices and variations in antibiotic selection among different hospitals. Selective pressure from the use of antimicrobial agents is a critical factor driving bacterial resistance. Therefore, it is crucial to exercise caution when using antimicrobial drugs in clinical settings to prevent the emergence of multidrug-resistant strains. To gain a deeper understanding of the genetic information and resistance profiles of *V. parahaemolyticus* in Nantong, whole-genome sequencing of clinical isolates will be a focal point of our future research endeavors.

Conclusion

To the best of our knowledge, this is a comprehensive research report on clinical isolates of *V. parahaemolyticus* in Nantong, providing a detailed analysis of the pathogen's antibiotic resistance and epidemiological characteristics through a large number of samples. A significant proportion of isolates (78.3%, 83/106) showed resistance to two or more antibiotics, and rational and cautious use of antibiotics is recommended for the treatment of *V. parahaemolyticus* infections. Spreading knowledge about preventing *V. parahaemolyticus* infection is crucial. For instance, it is advisable to avoid consuming raw seafood, especially during the summer, and choose thoroughly cooked food instead. PFGE analysis indicated that some isolates shared high homogeneity. Therefore, local health authorities should enhance market circulation and source supervision to prevent the possibility of sporadic cases or outbreaks.

Data Sharing Statement

The data generated in this study is confidential. For any inquiries, please contact the corresponding author.

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of Nantong Center for Disease Control and Prevention (approval 2023001). We obtained verbal consent from all patients or their guardians regarding the use of fecal samples, and strict confidentiality was maintained for all patient information.

Acknowledgments

We express our gratitude to the three sentinel hospitals in Nantong for providing the samples and sample information.

Funding

There is no funding to report.

Disclosure

The authors declare no conflicts of interest in this work.

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