Microbiology and Immunology

ORIGINAL ARTICLE

Virulence Potential of Nonclinical *Vibrio parahaemolyticus* Isolates From Vietnam: Evidence for Functional T3SS2-Mediated Enterotoxicity

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Received: 16 April 2025 | **Revised:** 20 May 2025 | **Accepted:** 24 May 2025

Funding: This study was supported in part by the Joint Usage/Research Center on Tropical Disease, Institute of Tropical Medicine, Nagasaki University (2021-Ippan-02, 2022-Ippan-06, 2023-Ippan-06) and the Japan Agency for Medical Research and Development (JP20wm0125006, JP223fa627004, JP22wm0225023, and JP23fk0108663). This study was also supported by a Grant for Joint Research Project of the Research Institute for Microbial Diseases, Osaka University (JRPRIMD24B8 and JRPRIMD24B10) and the Chemo-Sero-Therapeutic Research Institute.

Keywords: enterotoxicity | nonclinical isolate | pandemic strains | type III secretion system 2 (T3SS2) | *Vibrio parahaemolyticus*

ABSTRACT

Vibrio parahaemolyticus is a leading cause of seafood-borne gastroenteritis worldwide, and its pathogenic strains typically harbor thermostable direct hemolysin (TDH) and type III secretion system 2 (T3SS2). Although these virulence factors are associated primarily with clinical isolates, their presence in nonclinical environmental and food isolates raises concerns about their potential infection risk. In this study, we investigated the pathogenic potential of nonclinical *V. parahaemolyticus* isolates from Vietnam, which share serotypic and genotypic characteristics with pandemic strains. Serotyping and genetic analysis of 56 isolates (35 clinical and 21 nonclinical) revealed that two nonclinical isolates from shrimp and environmental water carried the *tdh* gene, T3SS2α genes, and pandemic markers that clustered phylogenetically with the pandemic strains. Protein expression assays confirmed that these isolates secreted TDH and the T3SS2 translocator (VopD2) at levels similar to those in the clinical reference strain. Bile exposure induced T3SS2-related gene expression, which suggests a conserved gene regulatory mechanism. Enterotoxicity evaluated using a rabbit ileal loop assay showed that two nonclinical isolates induced significant fluid accumulation. Genetic deletion and complementation experiments confirmed that T3SS2 was essential for enterotoxicity. These findings provide the first experimental evidence that nonclinical pandemic strains of *V. parahaemolyticus* possess functional enteric virulence mechanisms and suggest their potential as infection sources in endemic regions.

Abbreviations: KUT, K untypable; Vop, *Vibrio parahaemolyticus* outer protein.

Moses Lorenzo Akyeh and Masatomo Morita contributed equally to this study.

1 | Introduction

Vibrio parahaemolyticus is a halophilic gram-negative bacterium commonly found in marine and estuarine environments worldwide [1]. This pathogen is a major cause of foodborne gastroenteritis in humans, which can cause symptoms such as diarrhea and abdominal cramping after consumption of raw or undercooked seafood [2]. *V. parahaemolyticus* exhibits both serological and genetic diversity, and infections are often caused by a variety of strains and serotypes [3, 4]. In the past, *V. parahaemolyticus* infections were sporadic and confined primarily to Asia, but a significant epidemiological shift occurred in 1996 with the emergence of the O3:K6 serotype in India. This strain has since spread rapidly across the continent and has caused sporadic infections and outbreaks in areas where cases had been rare, such as South America and northern Europe, and has now become the predominant isolate in many areas. Following the spread of O3:K6, new serotype variants have emerged, including O1:KUT, O1:K25, and O4:K68 [5–7]. More recently, the O10:K4 serovar has emerged in China [8–10], where it is quickly becoming dominant, and strains of this serotype have also been detected in Thailand [11].

Several virulence factors have been identified, including two hemolysins, thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH), and two type III secretion systems (T3SSs: T3SS1 and T3SS2) [12]. T3SSs are specialized protein secretion apparatuses found in Gram-negative bacteria that allow for the direct injection of effector proteins into host cells. T3SS1 is present in all *V. parahaemolyticus* strains and is associated with cytotoxic activity against various cell lines [13]. By contrast, strains carrying hemolysins and the T3SS2 gene cluster are found primarily in isolates from people with diarrhea. The hemolysins and the T3SS2 gene cluster are encoded within a pathogenicity island known as the *Vibrio parahaemolyticus* pathogenicity island (Vp-PAI) [14].

T3SS2 is divided into two subtypes: T3SS2α, which is genetically linked to the *tdh* gene, and T3SS2β, which is associated with the *trh* gene [15]. Studies in animal models have demonstrated that T3SS2 plays a key role in enterotoxicity [16, 17]. The effectors VopV and TDH have been identified as the major contributors to T3SS2-dependent enterotoxicity, which underscores the importance of Vp-PAI in enteropathogenicity [18]. The genetic characteristics of pandemic strains are usually characterized by the presence of the *tdh* gene and T3SS2α subtype, as well as specific genetic markers such as *toxRS*/new sequence and/or the *orf8* gene [19–21].

Genomic comparisons of clinical and nonclinical isolates have shown greater serotype diversity in nonclinical strains and phylogenetic differentiation between clinical and environmental isolates [22]. Although epidemiological studies have shown that strains carrying these virulence factors are isolated predominantly from clinical cases, recent studies have isolated these strains from nonclinical sources, such as food and environmental water, and this raises concerns about their potential to cause infections [23–26]. However, despite research on the comparative virulence of several strains, no studies have examined specifically the pathogenic potential using gene-deficient strains, and it remains unclear whether and how these factors correlate with human pathogenicity. Therefore, the infection risk of environmental isolates remains unclear.

In this study, we investigated the virulence potential of nonclinical *V. parahaemolyticus* isolates obtained from environmental or food sources in Vietnam, an endemic region for *V. parahaemolyticus* infections. The serotype, genotype, and genomic lineage of these isolates are similar to those of pandemic strains. By analyzing the in vitro expression and secretion of virulence factors, as well as testing the in vivo pathogenicity using a rabbit ileal loop model, we report that these nonclinical isolates express functional T3SS2 protein, which is essential for induction of diarrhea. Our results provide the first experimental evidence that pandemic strains of *V. parahaemolyticus* isolated from environmental sources pose a significant risk for infection.

2 | Materials and Methods

2.1 | Bacterial Strains and Culture Conditions

The study included 56 *V. parahaemolyticus* strains collected in seven Vietnamese cities between 2009 and 2021; 35 were obtained from diarrheal stool, 13 from environmental water, and eight from food isolates including clam, shrimp, and fish (Table 1). The genome-sequenced pandemic strain RIMD2210633 (serotype O3:K6, *tdh*$^+$, T3SS2α) [14] and the non-pandemic *trh*-positive strain TH3996 (serotype O4:K1, *trh*$^+$, T3SS2β) [15] were also included as reference strains in the analysis. The Pathogenic Microbes Repository Unit, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, provided the reference strains. The *Escherichia coli* strains DH5α and SM10λ*pir* were used for DNA manipulation. *V. parahaemolyticus* and *E. coli* strains were grown in Luria–Bertani (LB) broth (1% tryptone and 0.5% yeast extract) containing 0.5% or 3% (w/v) NaCl at 37°C. The growth rates of all *V. parahaemolyticus* strains were monitored in LB medium, and it was confirmed that there were no significant differences in growth rates. The bacterial strains and plasmids used in this study are listed in Tables 2 and 3.

2.2 | Serotyping

The serotype was determined by conventional serological tests using a commercial test kit (Denka Seiken, Tokyo, Japan) containing 11 O antisera and 71 K antisera to evaluate lipopolysaccharide (O) and capsular (K) antigens, following the manufacturer's guidelines. Briefly, bacterial pellets from overnight cultures were suspended in 3% NaCl solution. The bacterial suspension was used for K-antigen testing. For O-antigen agglutination, suspensions were boiled for 2 h before O-antigen testing.

2.3 | Detection of Virulence-Associated Genes

Bacterial genomic DNA was extracted from 2.0 mL of overnight bacterial culture (about 10^9 colony-forming units/ml) using a DNeasy kit (Qiagen, Limburg, Netherlands) according to the manufacturer's instructions. DNA concentration was determined

TABLE 1 | Distribution of serotype, genotype, source, sample location, and year of isolation of Vietnam isolates.

ID	Serotype	Vp marker	Haemolysin gene		Pandemic marker		T3SS1-related gene				T3SS2α-related gene				T3SS2β-related gene				Source	Sample location	Year of isolation	Accession number
		Vp-toxR	*tdh*	*trh*	GS-PCR	*orf8*	*vscP*	*vopS*	*vscK*	*vscF*	*vopB2α*	*vscC2α*	*vscS2α*	*vopTα*	*vopB2β*	*vscC2β*	*vscS2β*	*vopCβ*				
VNVP001	O8:K41	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657095
VNVP002	O1:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657096
VNVP003	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657097
VNVP004	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657098
VNVP005	O10:K13	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Clam	Ho Chi Minh	2020	DRR657099
VNVP006	O3:K5	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Clam	Ho Chi Minh	2020	DRR657100
VNVP007	O5:K17	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Shrimp	Ho Chi Minh	2020	DRR657101
VNVP008	O1:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Shrimp	Ho Chi Minh	2020	DRR657102
VNVP009	O1:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Shrimp	Ho Chi Minh	2020	DRR657103
VNVP010	O4:K8	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657104
VNVP011	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657105
VNVP012	O1:K25	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657106
VNVP013	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657107
VNVP014	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657108
VNVP015	O2:K3	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657109

(Continues)

TABLE 1 | (Continued)

ID	Serotype	Vp marker	Haemolysin gene		Pandemic marker		T3SS1-related gene				T3SS2α-related gene				T3SS2β-related gene				Source	Sample location	Year of isolation	Accession number
		Vp-toxR	*tdh*	*trh*	GS-PCR	*orf8*	*vscP*	*vopS*	*vscK*	*vscF*	*vopB2α*	*vscC2α*	*vscS2α*	*vopTα*	*vopB2β*	*vscC2β*	*vscS2β*	*vopCβ*				
VNVP016	O4:K8	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657110
VNVP017	O1:K32	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657111
VNVP019	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657112
VNVP020	O10:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657113
VNVP022	O4:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657114
VNVP023	O4:K12	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657115
VNVP024	O4:K12	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657116
VNVP025	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Shrimp	Ben Tre	2020	DRR657117
VNVP026	O4:K8	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657118
VNVP027	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2021	DRR657119
VNVP029	O5:K17	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2021	DRR657120
VNVP030	O2:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2021	DRR657121
VNVP031	O4:KUT	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hai Phong	2013	DRR657122
VNVP032	O1:K25	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hai Phong	2013	DRR657123
VNVP033	O4:KUT	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hai Phong	2013	DRR657124
VNVP034	O5:K17	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657125
VNVP035	O5:K17	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657126
VNVP036	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2020	DRR657127

(Continues)

TABLE 1 | (Continued)

ID	Serotype	Vp marker	Haemolysin gene		Pandemic marker		T3SS1-related gene				T3SS2α-related gene				T3SS2β-related gene				Source	Sample location	Year of isolation	Accession number
		Vp-toxR	*tdh*	*trh*	GS-PCR	*orf8*	*vscP*	*vopS*	*vscK*	*vscF*	*vopB2α*	*vscC2α*	*vscS2α*	*vopTα*	*vopB2β*	*vscC2β*	*vscS2β*	*vopCβ*				
VNVP039	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2009	DRR657128
VNVP040	O4:K8	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2009	DRR657129
VNVP041	O4:K8	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2009	DRR657130
VNVP043	O10:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Fish	Ben Tre	2020	DRR657131
VNVP044	O3:K48	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Environmental water	Ho Chi Minh	2020	DRR657132
VNVP045	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Environmental water	Thai Binh	2020	DRR657133
VNVP047	O1:K49	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Environmental water	Thai Binh	2019	DRR657134
VNVP048	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Ben Tre	2010	DRR657135
VNVP049	O1:K32	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Fish	Ben Tre	2010	DRR657136
VNVP050	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Son La	2012	DRR657137
VNVP051	O10:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Environmental water	Hai Phong	2012	DRR657138
VNVP055	O4:K53	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	Environmental water	Thai Binh	2020	DRR657139
VNVP057	O10:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Environmental water	Thai Binh	2021	DRR657140
VNVP058	O4:K8	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2009	DRR657141
VNVP059	O4:K8	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2009	DRR657142

(Continues)

TABLE 1 | (Continued)

ID	Serotype	Vp marker	Haemolysin gene		Pandemic marker		T3SS1-related gene				T3SS2α-related gene				T3SS2β-related gene				Source	Sample location	Year of isolation	Accession number
		Vp-toxR	*tdh*	*trh*	GS-PCR	*orf8*	*vscP*	*vopS*	*vscK*	*vscF*	*vopB2α*	*vscC2α*	*vscS2α*	*vopTα*	*vopB2β*	*vscC2β*	*vscS2β*	*vopCβ*				
VNVP060	O5:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Environmental water	Thai Binh	2019	DRR657143
VNVP061	O10:KUT	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Environmental water	Thai Binh	2019	DRR657144
VNVP063	O5:K61	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	Environmental water	Thai Binh	2019	DRR657145
VNVP066	O3:K6	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	Diarrheal stool	Hanoi	2010	DRR657146
VNVP067	O4:K53	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	Environmental water	Thai Binh	2020	DRR657147
VNVP068	O4:K53	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	Environmental water	Thai Binh	2020	DRR657148
VNVP069	O4:K53	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	Environmental water	Thai Binh	2020	DRR657149
VNVP072	O4:K53	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	Environmental water	Thai Binh	2020	DRR657150

TABLE 2 | Bacterial strains used in this study.

Strain	Description	Source or references
Vibrio parahaemolyticus		
RIMD2210633	Pandemic clinical isolate; KP positive, serotype O3:K6, *tdh*$^+$	[14]
RIMD2210633Δ*vscN1*	T3SS1-deficient strain; *vscN1* deletion mutant (deletion from nt -225 to 1068 of the gene) derived from RIMD2210633	[16]
RIMD2210633Δ*vscN2*	T3SS2-deficient strain; in-frame *vscN2* deletion mutant (deletion from nt 160 to 978 of the gene) derived from RIMD2210633	[16]
RIMD2210633Δ*vscN1*Δ*vscN2*	T3SS1- and T3SS2-deficient strain; *vscN1* (deletion from nt -225 to 1068 of the gene) and *vscN2* (deletion from nt 160 to 978 of the gene) double-deletion mutant derived from RIMD2210633	[16]
VNVP025Δ*vscN1*	T3SS1-deficient strain; *vscN1* deletion mutant (deletion from nt -225 to 1068 of the gene) derived from WT VNVP025	This study
VNVP025Δ*vscN2*	T3SS2-deficient strain; in-frame *vscN2* deletion mutant (deletion from nt 160 to 978 of the gene) derived from WT VNVP025	This study
VNVP025Δ*vscN1*Δ*vscN2*	T3SS1- and T3SS2-deficient strain; *vscN1* (deletion from nt -225 to 1068 of the gene) and *vscN2* (deletion from nt 160 to 978 of the gene) double-deletion mutant derived from WT VNVP025	This study
VNVP045Δ*vscN1*	T3SS1-deficient strain; *vscN1* deletion mutant (deletion from nt -225 to 1068 of the gene) derived from WT VNVP045	This study
VNVP045Δ*vscN2*	T3SS2-deficient strain; in-frame *vscN2* deletion mutant (deletion from nt 160 to 978 of the gene) derived from WT VNVP045	This study
VNVP045Δ*vscN1*Δ*vscN2*	T3SS1- and T3SS2-deficient strain; *vscN1* (deletion from nt -225 to 1068 of the gene) and *vscN2* (deletion from nt 160 to 978 of the gene) double-deletion mutant derived from WT VNVP045	This study
TH3996	Clinical isolate; serotype O4:K1, *trh*$^+$	[15]
Escherichia coli		
DH5α	F$^-$ ϕ80Δ*lac*ZM15 Δ (*lacZYA argF*)*U169 deoP recA1 endA1 hsdR17* (r_K^- m_K^-)	Laboratory collection
SM10 *λpir*	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu *λpir* R6K	[27]

TABLE 3 | Plasmids used in this study.

Plasmid	Description	Source or references
Genetic manipulation		
pYAK1	R6K-*ori* suicide vector containing *sacB* gene	[16]
pYAK1-Δ*vscN1*	Derivative of suicide vector pYAK1 for generating the *vscN1* deletion mutants	[16]
pYAK1-Δ*vscN2*	Derivative of suicide vector pYAK1 for generating the *vscN2* deletion mutants	[16]
pSA19CP-MCS	Complement vector for *V. parahaemolyticus*, Cm^r	[16]
pSA19CP-*vscN2*	Derivative of suicide vector pYAK1 for complement of *vscN2*	[16]
CyaA translocation assay		
pHRP309	LacZ transcriptional fusion vector, Gmr	[28]
p309-Pro-*vtrB*	Derivative of pHRP309 containing *vtrB* promoter	[29]

using a NanoDrop spectrophotometer (Fisher Scientific, Massachusetts, USA). Virulence-associated genes were detected by polymerase chain reaction (PCR) as reported previously. PCR for *toxR* (*Vp-toxR*) was performed to identify *V. parahaemolyticus* [30]. PCR amplification of two hemolysins, *tdh* and *trh*, was performed as described [31]. PCR amplification of marker genes in pandemic strains (*toxRS*/new sequence and *orf8*) [19, 32], T3SS1 genes (*vscP*, *vopS*, *vscK*, and *vscF*) and two type T3SS2 genes (*vopB2α*, *vscC2α*, *vscS2α*, and *vopTα* for T3SS2α, and *vopB2β*, *vscC2β*, *vscSβ* and *vopC2β* for T3SS2β) [15, 31] was performed as described. The

primer sequences used in this study are listed in Table 4. All PCR analyses were performed on an Applied Biosystems Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, Massachusetts, USA). Amplicons from each PCR were separated on a 1.5% agarose gel containing GelGreen™ (Biotium Inc., CA, USA) to identify visually the presence or absence of amplification.

2.4 | Reversed Passive Latex Agglutination Assay

The production of TDH in culture supernatants was measured using a reverse passive latex agglutination assay (RPLA) kit (KAP-RPLA; Denka Seiken Co.) according to the manufacturer's instructions. Briefly, the culture supernatant obtained after

TABLE 4 | Primers used in this study.

Target gene	Primer name	Sequence 5′ > 3′	Amplicon size (bp)	References
Vp-toxR	*toxR*-F	GTCTTCTGACGCAATCGTTG	368	[30]
	toxR-R	ATACGAGTGGTTGCTGTCATG		
tdh	*tdh*-D3	CCACTACCACTCTCATATGC	251	[31]
	tdh-D5	GGTACTAAATGGCTGACATC		
trh	*trh*-F	TTGGCTTCGATATTTTCAGTATCT	484	[31]
	trh-R	CATAACAAACATATGCCCATTTCCG		
toxRS/new	GS-VP1	TAATGAGGTAGAAACA	651	[19]
	GS-VP2	ACGTAACGGGCCTACA		
orf8	*orf8*-F	AGGACGCAGTTACGCTTGATG	369	[33]
	orf8-R	CTAACGCATTGTCCCTTTGTAG		
vscP	VP1670 -F	ACCGATTACTCAAGGCGATG	392	[32]
	VP1670 -R	TACGTTGTTGGCGTGATTGT		
vopS	VP1686-F	CAAAAGCGATCACAAAAGCA	283	[32]
	VP1686-R	AGCGACTTAACGGCATCATC		
vscK	VP1689-F	AAGGTTGGCAAAAAGCGTTA	192	[32]
	VP1689-R	GCTCTTCAACGAGCCAAGAG		
vscF	VP1694-F	ACGATGCGACCAACAGTGTA	96	[32]
	VP1694-R	TTTTAATTGCATCGGTGACG		
vopB2α	VPA1362-F	CTGCAGGTATCGCATCTTCA	343	[32]
	VPA1362-R	TTAGAACCAACCGACGAAGC		
vscC2α	VPA1339-F	GATTCGCGGAACTCAAGAAG	250	[32]
	VPA1339-R	CTTGTCCGAGATCAACGTCA		
vscS2α	VPA1335-F	ATGTAACGGCGGCTAGCTTA	174	[32]
	VPA1335-R	CAAACTGTGTCAGTAGCACCA		
vopTα	VPA1327-F	TGGCGAAAGAGCCATTAGAT	97	[32]
	VPA1327-R	TCAACTCCAAATTCGCCTTC		
vscC2β	*vscC2*-F	GTACTTTGCTGTCTAACC	1400	[15]
	vscC2-R	CTTACTCTTAACTTCCGACG		
vopB2β	*vopB2*-F	GAGCCTGTTGCTCTATGGAGCCAGG	942	[15]
	vopB2-R	CGACACAGAACGCAATGCTTGCTCG		
vopCβ	*vopC*-F	AACCAACTTGCGACTAAATC	594	[15]
	vopC-R	TCCCGACAGTTTTTCTGCAC		
vscS2β	*vscS2*-F	TTGATGTTGTTTCGGCTAGC	224	[15]
	vscS2-R	CCACCGCCGAACTCGGCTAACAAG		
vscN1	Δ*vscN1*check-F	TCAAATACGCTTGAACTGAGCGATGGTCG	2019 (intact)	This study
	Δ*vscN1*check-R	AACGTGAATGTCCGCTACCGAGCGTTCTGC	726 (deletion)	
vscN2	Δ*vscN2*check-F	CGGTGAAATCGTTAAGGTGACAGGCTC	1245 (intact)	This study
	Δ*vscN2*check-R	CGATAGCTCCCACTTTTGACTCAAGCGAC	772 (deletion)	

incubation with overnight shaking at 200 rpm at 37°C in 3% NaCl LB broth in the presence or absence of 0.04% crude bile and then twofold serially diluted in phosphate buffer [pH 7.0] containing 0.5% bovine serum albumin and 0.1% sodium azide in 96-well V-shaped bottom microplates (Greiner Bio-One, Frickenhausen, Germany). Sensitized latex anti-hemolytic toxin was added to each well, and the mixture was mixed thoroughly and then incubated at room temperature (23°C) for 24 h. The aggregation of latex particles in the wells was determined visually. Non-sensitized latex and control heat-stable hemolytic toxin were used as negative and positive controls, respectively.

2.5 | Whole-Genome Sequencing and Genome Assembly

Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen), and the concentrations were determined using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific). The genomic DNA libraries were prepared using the QIAseq FX DNA library kit (Qiagen) according to the manufacturer's instructions and sequenced on a MiSeq (Illumina) with 300 bp paired-end reads. Genome assembly was performed using SPAdes v.3.13.0, with the "-careful" and "--cov-cutoff auto" options [34]. The genome assemblies were annotated using the DDBJ Fast Annotation and Submission Tool (https://dfast.ddbj.nig.ac.jp/) [35].

2.6 | Phylogenetic Analysis

We carried out a pan-genome analysis of the 56 *V. parahaemolyticus* genomes from Vietnam and the reference genome of *V. parahaemolyticus* strain RIMD2210633 [14]. Core gene alignments were constructed using Panaroo (v.1.5.0) with the "--clean-mode strict", "--merge_paralogs", "-a core" and "--aligner mafft" options [36]. Single nucleotide variants (SNVs) were extracted from the core gene alignment using SNP-sites (v2.5.1) and were used to identify phylogenetic relationships by reconstructing the phylogenetic tree using IQ-TREE (v.2.1.2) with 1,000 ultrafast bootstrap replicates [37, 38]. The phylogenetic tree was visualized using iTOL [39].

2.7 | Protein Sample Preparation

The production and secretion of TDH, VopD1, and VopD2 were analyzed using immunoblotting as previously described [40, 41]. Briefly, overnight cultures were diluted 100-fold in LB broth containing 0.5% NaCl with or without 0.04% crude bile and incubated with shaking at 200 rpm for 5 h at 37°C. After incubation, the bacterial cultures were centrifuged to separate the bacterial pellet and supernatant. Bacterial pellets were solubilized in Laemmli buffer. Secreted proteins were precipitated by adding ice-cold trichloroacetic acid at a final concentration of 10% (v/v) on ice for 1 h, followed by centrifugation at 15,000 ×g for 30 min at 4°C. The pellets were rinsed with cold acetone, solubilized in Laemmli buffer, and denatured at 95°C for 5 min. Samples for western blot analysis were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

2.8 | Immunoblotting

Protein samples separated by SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane by semidry electroblotting. The transferred membranes were blocked in TBST (20 mM Tris-HCl at pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk for 1 h and probed with anti-VopD1, anti-VopD2, and anti-TDH rabbit polyclonal antibodies, and anti-RNA polymerase beta rabbit monoclonal antibody (Abcam plc., Cambridge, UK), followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (Invitrogen, CA, USA). The blots were developed using enhanced chemiluminescence (ECL) (GE HealthCare, IL, USA), and images were captured using LuminoGraph II (ATTO, Tokyo, Japan).

2.9 | Construction and Confirmation of Gene-Deletion Mutant Strain

V. parahaemolyticus strains (VNVP025 and VNVP045) were conjugated with *E. coli* SM10λ*pir* haboring the pYAK-Δ*vscN1* or pYAK-Δ*vcsN2* plasmid. The conjugates were selected on thiosulfate citrate bile sucrose agar containing 5 μg/mL chloramphenicol, and then counterselection for mutants by induction with LB broth supplemented with 10% sucrose. The desired gene deletion was confirmed by PCR using the primers listed in Table 4.

2.10 | β-Galactosidase Reporter Gene Assay

V. parahaemolyticus strains harboring pHRP309 or pHRP309-*vtrB* reporter plasmids were grown in LB broth containing 0.5% NaCl with or without 0.04% crude bile with shaking at 200 rpm for 3 h at 37°C. The β-galactosidase activity of the bacterial cell lysates was measured using Miller's method with the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG), as described previously [42].

2.11 | Rabbit Ileal Loop Test

Rabbit ileal loop tests were performed as previously described [16]. Briefly, overnight cultures of *V. parahaemolyticus* were diluted 100-fold in LB broth containing 0.5% NaCl and grown with shaking for 5.5 h. After incubation, bacteria were harvested by centrifugation and suspended in LB broth containing 0.5% NaCl. One milliliter of the bacterial suspension (2×10^9 colony-forming units/mL) was injected into the ligated ileal loops of a 1.5-kg female New Zealand White rabbit (the length of a loop was approximately 8 cm), and fluid accumulation (FA) in each loop was measured 18 h after challenge. The FA ratio represents the amount of accumulated fluid (mL) per length of ligated rabbit small intestine (cm).

3 | Results

3.1 | Serotyping and Virulence Gene Profiling of *V. parahaemolyticus* Isolated in Vietnam

A total of 56 Vietnamese *V. parahaemolyticus* strains were analyzed for serotype, hemolysin genes, pandemic markers, and

TABLE 5 | Distribution of serotypes and genotypes of Vietnamese isolates by origin.

Serotype	Number of strains	Vp marker	T3SS1-related gene				Haemolysin gene		T3SS2α-related gene				T3SS2β-related gene				Pandemic marker	
		Vp-toxR	*vscP*	*vopS*	*vscK*	*vscF*	*tdh*	*trh*	*vopB2α*	*vscC2α*	*vscS2α*	*vopTα*	*vopB2β*	*vscC2β*	*vscS2β*	*vopCβ*	GS-PCR	*orf8*
Clinical																		
O1:KUT	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O1:K25	1	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	+	−
O1:K25	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O1:K32	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O2:KUT	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O2:K3	1	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	+	−
O3:K6	12	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	+	−
O4:KUT	2	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	+	−
O4:KUT	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O4:K8	7	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	+	−
O4:K12	2	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O5:K17	3	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O8:K41	1	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	+	−
O10:KUT	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Number of strains	**35**	**35**	**35**	**35**	**35**	**35**	**24**	**0**	**24**	**24**	**24**	**25**	**0**	**0**	**0**	**0**	**24**	**0**
Noncli-nical																		
O1:KUT	2	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O1:K32	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O1:K49	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O3:K5	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O3:K6	2	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	+	−
O3:K48	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
O4:K53	5	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−
O5:KUT	1	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−

(Continues)

TABLE 5 | (Continued)

Number of strains	35	35	35	35	35	24	0	24	24	25	0	0	0	24	0
O5:K17	1	+	+	+	+	−	−	−	−	−	−	−	−	−	−
O5:K61	1	+	+	+	+	−	−	−	−	−	−	−	−	−	−
O10:KUT	4	+	+	+	+	−	−	−	−	−	−	−	−	−	−
O10:K13	1	+	+	+	+	−	−	−	−	−	−	−	−	−	−
Number of strains	**21**	**21**	**21**	**21**	**21**	**2**	**0**	**2**	**2**	**2**	**5**	**5**	**5**	**2**	**0**

T3SS-associated genes. These strains were isolated between 2009 and 2021 from diverse sources in Vietnam, including diarrheal stool (35 isolates), environmental water (13 isolates), and food samples such as shrimp, fish, and clams (8 isolates) (Table 1). The results from Table 1 were reorganized by isolate origin, serotype, and gene profile, and are summarized in Table 5. The serotype distribution among Vietnamese *Vibrio parahaemolyticus* isolates reveals notable differences between clinical and nonclinical sources. Among the clinical isolates ($n = 35$), the most dominant serotype was O3:K6, found in 12 isolates. This serotype is widely recognized as a pandemic clone and is frequently associated with human diarrheal disease worldwide. The high prevalence of O3:K6 and related serotypes among clinical samples highlights their significant role in *V. parahaemolyticus* infections in Vietnam. In contrast, the nonclinical isolates obtained from environmental sources, such as seafood and surface water, showed greater serotype diversity. Unlike the clinical group, which was dominated by O3:K6, no single serotype predominated among the nonclinical isolates. This distribution reflects the broad diversity of *V. parahaemolyticus* serotypes in the Vietnamese environment, many of which appear to have limited association with human disease.

The presence of the species-specific marker *toxR* (*Vp-toxR*) was confirmed in all isolates. The Type III Secretion System 1 (T3SS1)-related genes (*vscP*, *vopS*, *vscK*, and *vscF*) were universally present in both clinical and nonclinical isolates, suggesting their conservation and essential role in the biology of the species. *tdh*-positive isolates accounted for most of the clinical isolates, whereas *trh*-positive isolates were absent in both the clinical and nonclinical isolates. All *tdh*-positive strains harbored the alpha-type T3SS2 genes. More than half of the *tdh*-positive strains were positive for pandemic markers, and their serotypes were O3:K6 and O1:K25, indicating that a significant proportion of clinical cases were caused by pandemic-associated lineages. Most of the nonclinical isolates were negative for the hemolysin genes, and only two were positive for the *tdh* gene. Interestingly, these *tdh*-positive nonclinical isolates were also positive for both the pandemic marker and the T3SS2α genes, and their serotype was O3:K6, which was predominant in the clinical pandemic strains. In addition, they were obtained from different sources (VNVP025 from food [shrimp], and VNVP045 from environmental water) and different locations (Table 1). These results indicate that *V. parahaemolyticus* diarrhea caused by the pandemic strain is predominant in Vietnam and that strains with serotypes and gene profiles similar to those of the pandemic strain may be present in the Vietnamese environment.

3.2 | Phylogenetic Analysis

The PCR method used in this study to analyze pandemic markers has previously been applied in molecular epidemiological studies of *V. parahaemolyticus*. However, the existence of pandemic strains lacking marker genes and non-pandemic strains positive for marker genes has also been reported [24, 43], and these PCR methods for pandemic markers may not be applicable to all pandemic strains. Therefore, we conducted a phylogenetic analysis using 80,344 SNVs on concatenated core genes in all Vietnamese isolates and the pandemic reference strain RIMD2210633 to identify which isolates belong to the pandemic clade (Figure 1). Isolates were generally clustered by

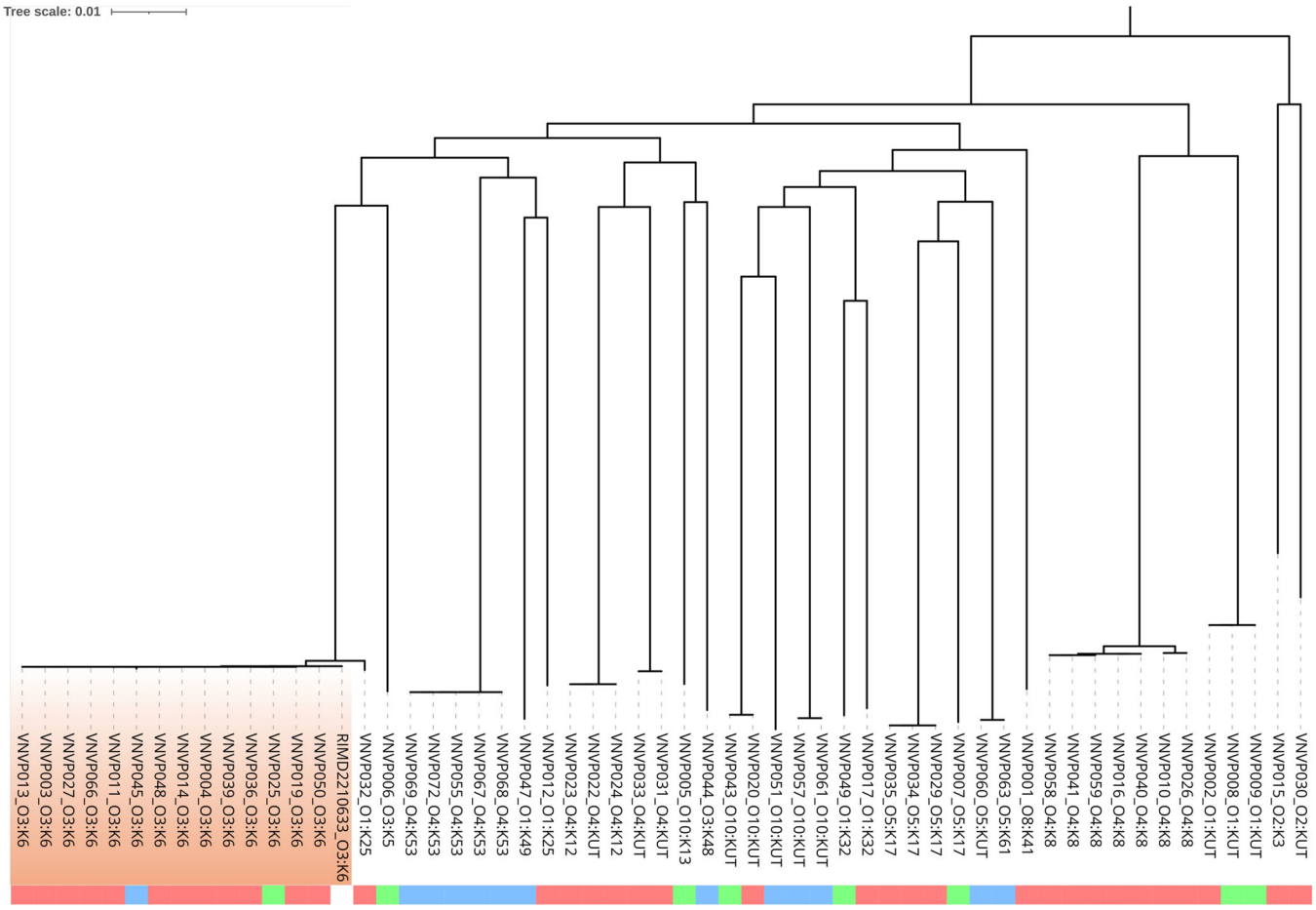

FIGURE 1 | A midpoint-rooted phylogenetic tree of *V. parahaemolyticus* isolates in Vietnam**.** The serotype is indicated after the strain name and the sources of isolates are represented in colored boxes (red: clinical sample, green: food, blue: environmental water). The clade containing the pandemic reference strain RIMD2210633 is highlighted in orange.

serotype, and the clade containing the pandemic reference strain RIMD2210633 included two nonclinical and 12 clinical isolates, all of which had the O3:K6 serotype and were positive for all pandemic markers, confirming their phylogenetic placement within the pandemic lineage. Of the two nonclinical isolates, one was isolated from environmental water and the other from food. These findings indicate that strains with a very close genetic background to the pandemic strains were present in the Vietnamese aquatic environment and contaminated food.

Conversely, isolates located outside the orange-highlighted pandemic clade formed distinct lineages, many of which are associated with the environment. These strains exhibit greater genetic divergence from the pandemic clade, implying they may represent endemic or environmentally adapted populations of *V. parahaemolyticus*. Overall, the phylogenetic analysis reveals a mixed population structure in Vietnam, characterized by the co-circulation of both pandemic and non-pandemic lineages.

3.3 | Production and Secretion of TDH, T3SS1, and T3SS2-Related Proteins in Nonclinical Isolates of *V. parahaemolyticus* From Vietnam

Numerous cases of *V. parahaemolyticus* carrying pathogenic factors have been isolated from nonclinical specimens [22–26]. However, few reports have analyzed the production of these factors at the protein level. Therefore, to investigate whether the VNVP025 and VNVP045 strains, which were isolated from shrimp and environmental water, could produce and secrete TDH and T3SS1- and T3SS2-related proteins, we used immunoblotting analysis to examine the bacterial pellets and culture supernatants (Figure 2A).

VopD1 and VopD2 are translocators of T3SS1 and T3SS2, respectively, and both are secreted in a T3SS-dependent manner [16]. Although VNVP025 exhibited slightly reduced VopD1 production and secretion compared to other strains, both VNVP025 and VNVP045 showed robust secretion of TDH and VopD2, closely resembling the secretion profile of the pandemic clinical strain RIMD2210633. These findings indicate that the nonclinical isolates retain functionally conserved T3SS2-mediated virulence mechanisms comparable to those observed in clinical pandemic strains.

To determine whether T3SS1 and T3SS2 of the nonclinical strains are functionally involved in the secretion of VopD1 and VopD2, gene-deletion mutants with dysfunctional T3SS1 and T3SS2 were generated by deleting the *vscN1* and *vscN2* genes, respectively. *vscN1* and *vscN2* encode ATPases essential for the secretory activity of T3SS1 and T3SS2, respectively [16]. The deletion of each gene was confirmed by PCR (Figure 2B).

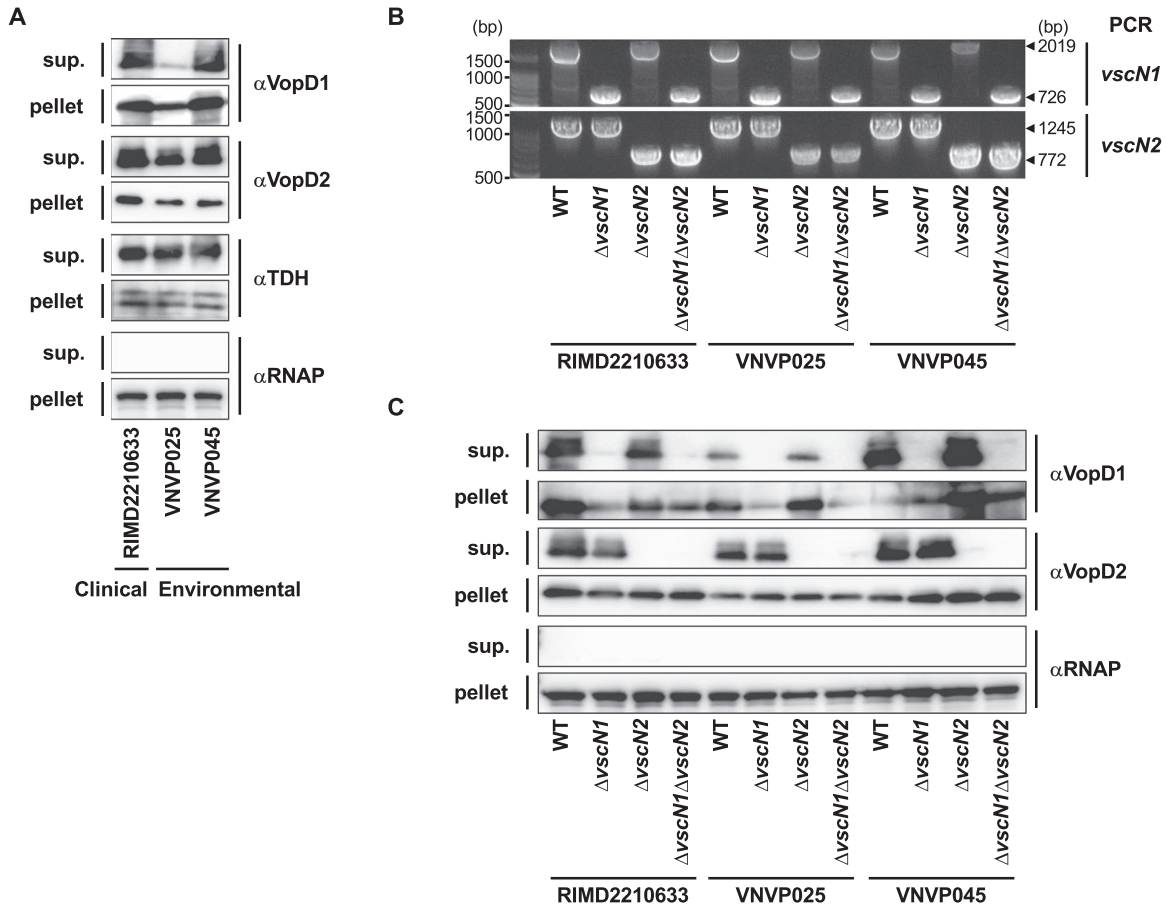

FIGURE 2 | Production and secretion of TDH, T3SS1, and T3SS2-related proteins in Vietnamese nonclinical isolates of *V. parahaemolyticus*. A. Immunoblotting of TDH T3SS1- and T3SS2-related proteins in the bacterial pellet and culture supernatants of clinical (RIMD2210633) and Vietnamese nonclinical isolates (VNVP025 and VNVP045) of *V. parahaemolyticus*. The bacterial pellet (pellet) and culture supernatant (sup.) were separated by SDS-PAGE and blotted with anti-TDH, anti-VopD1, and anti-VopD2 antibodies. Anti-RNA polymerase antibody (αRNAP) was used as a loading control. B. Construction and confirmation of T3SS1- and T3SS2-deficient mutants by PCR. Gene deletions were confirmed by the size of the amplified PCR products targeting the *vcsN1* and *vscN2* genes. The amplified PCR products of the *vscN1* and *vscN2* genes were 2019 bp for full-length and 726 bp for gene deletions of *vcsN1*, and 1245 bp for full-length and 772 bp for gene deletions of *vscN2*. C. Immunoblotting of VopD1 and VopD2 proteins in the bacterial pellet and culture supernatants of T3SS1-deficient (Δ*vscN1*) and/or T3SS2-deficient (Δ*vscN2*) strains. The bacterial pellet (pellet) and culture supernatant (sup.) were separated by SDS-PAGE and blotted with the anti-VopD1 and anti-VopD2 antibodies. Anti-RNA polymerase antibody (αRNAP) was used as a loading control.

The effect of these deletions on VopD1 and VopD2 secretion was confirmed by immunoblotting (Figure 2C). The immunoblotting results showed that each gene deletion disrupted the secretion of its respective protein but did not affect the secretion of the other protein. These results indicate that the nonclinical isolates express functional T3SSs and secrete their respective substrates.

3.4 | Upregulation of the Bile-Mediated VtrABC Transcriptional Regulatory System in Nonclinical Isolates of *V. parahaemolyticus* From Vietnam

T3SS2 is a major contributor to enterotoxicity in *V. parahaemolyticus* and its gene expression is induced by bile acids [29, 44, 45], which are abundant in the human intestinal tract. The VtrABC transcriptional regulatory system mediates the induction of T3SS2 gene cluster expression by bile acids (Figure 3A). The VtrAC complex senses bile acid stimulation and induces the expression of VtrB, which in turn induces the expression of Vp-PAI genes, including the T3SS2 gene cluster and *tdh*. This transcriptional cascade is essential for the induction of enterotoxicity.

To investigate whether the nonclinical isolates of *V. parahaemolyticus* possess a VtrABC-mediated mechanism for the induction of Vp-PAI gene expression by bile, we used a reporter assay with the *vtrB* promoter (Figure 3B). The β-galactosidase activity of the nonclinical strains was as low as that of the empty vector in the absence of bile, but its activity increased significantly in the presence of bile in a pattern similar to that of the RIMD2210633 strain.

The induction of TDH production by bile was also observed using KAP-RPLA (Figure 3C). Similarly, the induction of VopD2 and TDH production by bile was confirmed by immunoblotting (Figure 3D). These results indicate that nonclinical strains have a mechanism for inducing the expression of Vp-PAI genes that is similar to that in the clinical isolates.

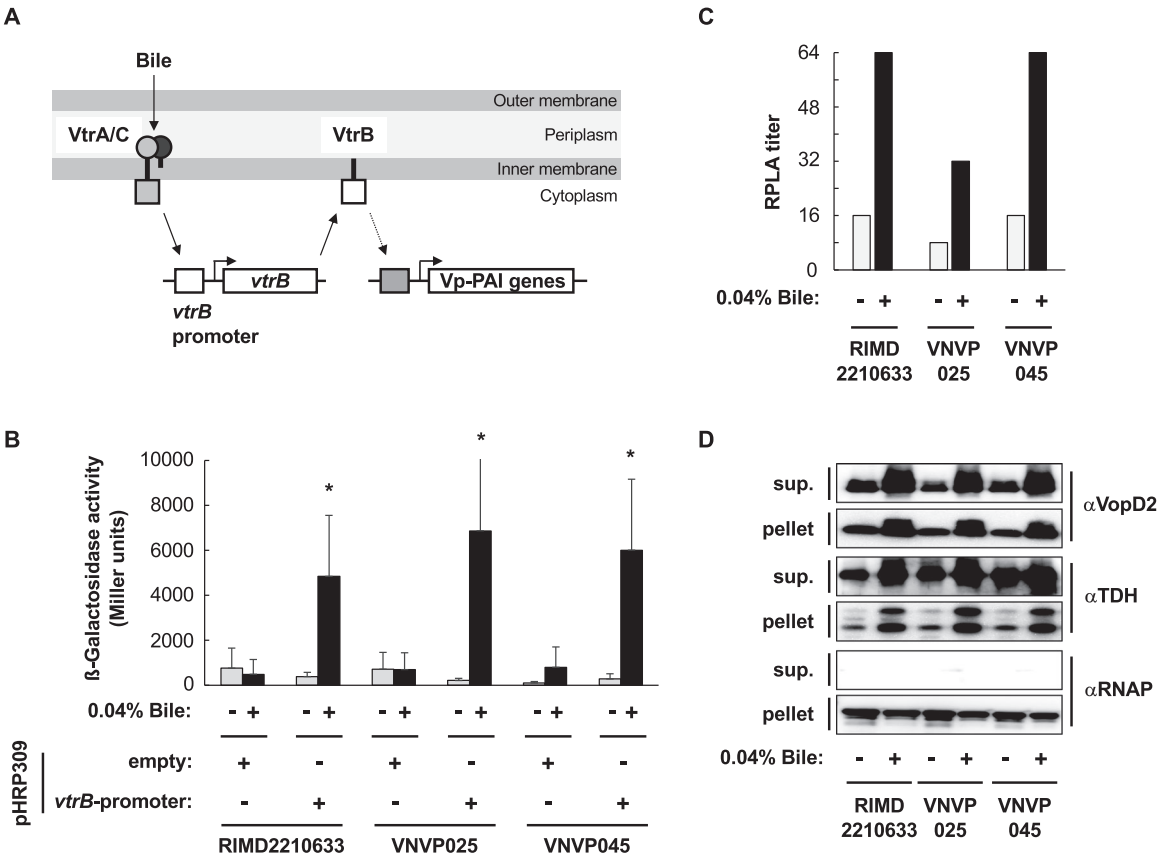

FIGURE 3 | Upregulation of the bile-mediated VtrABC transcriptional regulatory system in Vietnamese nonclinical isolates of *V. parahaemolyticus*. A. Diagram of the T3SS2 transcriptional regulatory system. Bile activates the VtrAC complex to induce *vtrB* expression, and VtrB activates Vp-PAI gene expression. B. β-Galactosidase activity of the β-galactosidase reporter gene under the *vtrB* promoter (pHRP309-*vtrB* promoter) in *V. parahaemolyticus*. The bars represent the average of three independent experiments. Error bars indicate the standard deviation (SD). Asterisks indicate significant differences from the results without crude bile ($p < 0.01$). C. Bile acid-induced increase in TDH production in the culture supernatant. TDH secretion in the presence or absence of 0.04% crude bile was quantified using reversed passive latex agglutination (RPLA). D. Production and secretion of VopD2 and TDH from *V. parahaemolyticus* cultured in LB broth with or without 0.04% crude bile. Anti-RNA polymerase antibody (αRNAP) was used as a loading control.

3.5 | T3SS2-Mediated Enterotoxic Activity of Nonclinical Isolates of *V. parahaemolyticus* From Vietnam

Only a few reports have examined enterotoxic activity in nonclinical isolates [23, 25]. However, in these reports, the presence of virulence factors did not necessarily correlate with pathogenicity, and the factors contributing to enterotoxic activity have not been identified as the gene-deletion strains of virulence factors have not been examined. Thus, the mechanism of diarrhea induction by nonclinical strains remains unclear.

To determine whether nonclinical strains exhibit enterotoxic activity and whether T3SS2 is involved in this activity, the enterotoxicity of T3SS gene-deletion mutants of nonclinical strains was evaluated using the rabbit ileal loop test and compared to the pandemic clinical strain RIMD2210633 (Figure 4A,B). WT nonclinical isolates induced the same level of FA as the clinical strains. This activity was significantly decreased by *vscN2* deletion (Δ*vscN2*), which disrupts T3SS2 function, but was unaffected by *vscN1* deletion (Δ*vscN1*), which disrupts T3SS1 function.

Next, a *vscN2* gene complementation strain was constructed to examine the effects on VopD2 secretion and enterotoxicity. The secretory activity of VopD2 and enterotoxic activity of the WT VNVP025 strain, which was reduced by deletion of the *vscN2* gene (VNVP025Δ*vscN2*), were significantly restored by complementation with the *vscN2* gene (VNVP025Δ*vscN2*/pSA19CP-*vscN2*) (Figure 4C–E). This finding indicates that T3SS2-positive nonclinical strains exhibit T3SS2-mediated enterotoxic activity.

4 | Discussion

V. parahaemolyticus is a major seafood-borne pathogen that causes acute gastroenteritis worldwide [1, 2]. Virulence factors associated with cytotoxicity and enterotoxicity have been identified in clinical isolates of *V. parahaemolyticus*, and similar factors have also been detected in nonclinical isolates [23–26], and these raise concerns about their potential as sources of human infection. In this study, we focused on pandemic strains isolated from nonclinical samples in Vietnam and evaluated their pathogenic potential.

Studies of the prevalence of *V. parahaemolyticus* in Vietnam have reported significant contamination of environmental

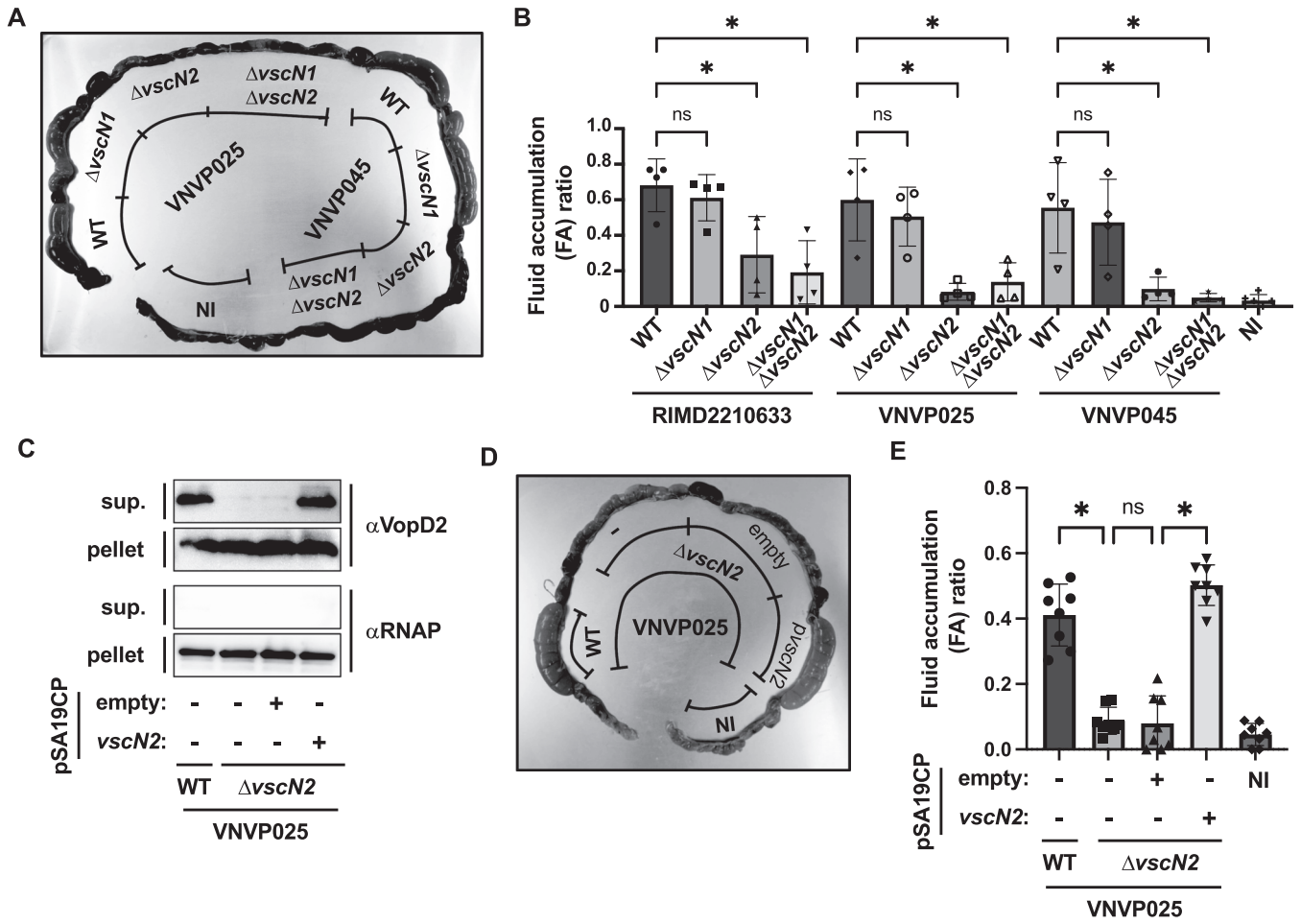

FIGURE 4 | T3SS2-mediated enterotoxic activity of Vietnamese nonclinical isolates of *V. parahaemolyticus*. A. A rabbit ileal loop test was used to measure the enterotoxic activity of Vietnamese nonclinical isolates of *V. parahaemolyticus*. The isolates and their derivative strains were injected separately into the loops. B. Fluid accumulation (FA) in the rabbit ileal loop test. FA was calculated as the amount of accumulated fluid (in ml) per length (in cm) of the ligated rabbit small intestine. The bars represent the average of independent experiments ($n = 4$). Error bars indicate the SD. Asterisks indicate significant differences from the results obtained with the WT strains ($p < 0.01$). C. Immunoblotting of VopD2 protein in the bacterial pellet and culture supernatants of the *vscN2* deletion strain of VNVP025 (VNVP025Δ*vscN2*) and its complementary strain (VNVP025Δ*vscN2*/pSA19CP-*vscN2*). D. A rabbit ileal loop test was used to measure the enterotoxic activity of the *vscN2* deletion strain of VNVP025 and its complementary strain. E. Enterotoxic activity of VNVP025Δ*vscN2* and VNVP025Δ*vscN2*/pSA19CP-*vscN2* analyzed in the rabbit ileal loop test. The bars represent the average of independent experiments ($n = 8$). The error bars indicate the SD. Asterisks indicate significant differences from the results obtained for the WT VNVP025 and VNVP025Δ*vscN2* ($p < 0.01$).

water and seafood distributed in retail markets [46–49]. PCR-based analyses of isolates from these sources have detected the presence of the hemolysin genes, *tdh* and/or *trh*, which indicates the existence of pathogenic strains in the environment [48, 50]. A serotyping study by Tran et al. identified pandemic marker (GS-PCR)-positive serotype O3:K6 strains in nonclinical samples collected in 2015 and 2016, which raises concerns about the spread of pandemic strains in environmental sources [50]. Notably, pandemic strains were detected in clinical isolates as far back as 1997, and a serovar transition accompanied this pandemic outbreak [43].

Few studies have characterized the serotypes or genotypes of *V. parahaemolyticus* strains isolated in Vietnam. In this study, we analyzed isolates collected primarily between 2019 and 2021, and we found that pandemic O3:K6 strains continue to be prevalent (Tables 1 and 5). Although *tdh*-positive strains were rare among nonclinical isolates, phylogenetic analysis revealed that some nonclinical isolates shared a high degree of genetic similarity with known pandemic strains (Figure 1).

Previous studies of *V. parahaemolyticus* pathogenicity have used primarily clinical pandemic strains, with the first complete genome reported in 2003 [14]. The strain has been studied extensively using rabbit ileal loop assays and other models [16, 18, 40]. By contrast, few studies have assessed the pathogenicity of nonclinical isolates. Some have evaluated cytotoxicity and invasiveness in Caco-2 cells [24], enterotoxicity in rabbit ileal loop model [23], colonization in infant rabbit models [25], or lethality in mouse and Galleria mellonella larvae models [26]. However, these studies have not identified the specific virulence factors responsible for such phenotypes using mutant strains, which leaves the pathogenic potential of nonclinical isolates open to debate.

In this study, we characterized the serotypes and genotypes of clinical and nonclinical isolates from Vietnam and assessed the

enterotoxicity of nonclinical strains harboring virulence factors. We found that nonclinical pandemic strains in Vietnam express functional T3SS1 and T3SS2 systems with secretion activity similar to that of clinical isolates. We also confirmed the expression and secretion of TDH. Although the bile-induced upregulation of *tdh* and T3SS2 gene expression has been reported previously in clinical strains [29, 44], our findings suggest that similar regulatory mechanisms exist in nonclinical pandemic strains.

The rabbit ileal loop assays revealed that nonclinical isolates exhibited enterotoxic activity similar to that of clinical strains. The dependence of this activity on T3SS2 provides evidence for the potential of these strains as enteropathogens. Although previous reports have identified virulence gene-positive *V. parahaemolyticus* strains in nonclinical sources, direct evaluation of their pathogenicity has been scarce. The current study provides the first direct evidence that at least some nonclinical pandemic O3:K6 strains in Vietnam possess pathogenic mechanisms equivalent to those of clinical isolates. These findings underscore the potential public health risk posed by nonclinical *V. parahaemolyticus* strains and highlight the importance of continuous surveillance of environmental isolates, particularly with respect to their virulence-related traits.

Author Contributions

Conceptualization: Toshio Kodama. Data curation: Masatomo Morita and Toshio Kodama. Formal analysis: Masatomo Morita and Toshio Kodama. Funding acquisition: Masatomo Morita and Toshio Kodama. Investigation: Masatomo Morita, Toshio Kodama and Moses Lorenzo Akyeh. Methodology: Masatomo Morita and Toshio Kodama. Resources: Pham Hong Quynh Anh, Taichiro Takemura, Pham Tuyet Ngoc Linh, Nguyen Dong Tu, Kazuhisa Okada, and Toshio Kodama. Supervision: Toshio Kodama. Validation: Toshio Kodama. Visualization: Masatomo Morita and Toshio Kodama. Writing original draft preparation: Masatomo Morita and Toshio Kodama. Review and editing: Masatomo Morita, Moses Lorenzo Akyeh, Sarunporn Tandhavanant, Pham Hong Quynh Anh, Hiroyuki Terashima, Pham Tuyet Ngoc Linh, Nguyen Dong Tu, Taichiro Takemura, Hiroyuki Terashima, Hirotaka Hiyoshi, Kazuhisa Okada, and Toshio Kodama.

Ethics Statement

This study did not include patient information, and the animal experiments adhered to an experimental protocol approved by the Guidelines for the Care and Use of Laboratory Animals at Nagasaki University, Nagasaki, Japan (approval number 2303221848).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions. Short-read sequence data were submitted to the DDBJ Sequenced Read Archive, and the accession numbers are listed in Table 1.

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