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PREVALENCE OF HEAT-RESISTANT *VIBRIO* PARAHAEMOLYTICUS IN RETAIL SEAFOOD

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ABSTRACT

Previous work using a modified two-step enrichment method recovered total *Vibrio parahaemolyticus*, including the regular and heat/pasteurization-resistant strains. These strains would remain undetectable by exclusive cultivation protocol, attributed to their viable-but-non-culturable (VBNC) property, from environmental shellfish seafood. Hence, this suggests the need to validate retail seafood safety from this human pathogen and reform the inspection examination and cooking protocols mandated by the regulators to mitigate vibriosis recurrence. This modified method was further explored with raw, frozen shellfish seafood products from retail stores nationwide in the US for heat/pasteurization-resistant *V. parahaemolyticus* using nourishment only (one-step enrichment) or nourishment and heating selection (two-step enrichment) combined, followed by selective cultivation on thiosulphate-citrate-bile-salt agar (TCBS) media. Both presumptive regular and heat-resistant *V. parahaemolyticus* were recovered from processed shrimps (deveined/non-deveined, shell, and tail on), and non-processed oysters and crabs. Of 29 isolates recovered, nine (31%) were confirmed as heat-resistant (two from oyster; 7%) and regular (seven, two from shrimp; five from oyster; 24%) *V. parahaemolyticus* using 16S rDNA bacterial identification. However, none of them were resistant to pasteurization conditions. Virulence phenotype and genotype examinations indicated the presence of hemolysis- and urease-positive *V. parahaemolyticus* and that their unparallel phenotype/genotype correlation could be attributed to gene manipulation. For the first time, the present study reveals the prevalence of total *V. parahaemolyticus*, both regular and heat-resistant pathogenic strains, in retail shellfish products using the modified two-step enrichment technique

and acclaims the need to reform the investigative and cooking standard protocol of USDA and other food safety regulatory entities.

Keywords: Foodborne pathogen, Heat-resistant, Pasteurization-resistant, Seafood safety, Viable-but-non-culturable (VBNC), *Vibrio parahaemolyticus*, Vibriosis.

1. INTRODUCTION

V. parahaemolyticus has existed as a Gram-negative shellfish symbiont (1), foodborne bacterial pathogen with biological properties non-compliant with food safety protocol, such as viable-but non-culturable (VBNC) (2). Infections could be acquired frequently during warm seasons (3-5) through ingestion of raw or undercooked contaminated seafood and open wounds, leading to gastroenteritis, bacteremia, fever, vomiting, and potential death (6,7).

This pathogen could possess virulence determinants including urease (8) and one or more hemolysins (i.e., thermostable direct hemolysin, *tdh*; thermostable related hemolysin, *trh*; thermolabile hemolysin, *tlh*) (9), and the hemolysin phenotype in resuscitated *V. parahaemolyticus* could be arbitrarily assessed using Kanagawa phenomenon test (8,9) because of the frequent inconsistent test result and gene availability correlation (10). Recently, Meza et al. (8) recovered a group of VBNC *V. parahaemolyticus* distinctive by their ability to withstand high isolation temperature (i.e., 80 °C) and pasteurization resistance characterization temperature (i.e., 65 °C) (8), and further biochemical investigations revealed that they could be equally pathogenic as the clinical isolates; hence, posing a novel threat to food safety from this pathogen.

Inevitable, controlled use of antibiotics, including the therapeutic antibiotics tetracycline and quinolones (11), in seafood farming is positively impacting the emergence of antibiotic resistance and multiple antibiotic resistance (MAR) of bacteria, including *V. parahaemolyticus* (12), in farm-raised seafood organisms as well as the wild-caught organisms (13). This is particularly prevalent in farm-raised seafood (13). Ampicillin-resistant *V. parahaemolyticus* is widely prevalent in environmental, retail, and farm-raised isolates, as described previously by Meza et al. (8), Letchumanan et al. (12), and Sharma et al. (13), respectively, leading to penicillin therapeutic complications (11). Further, Meza et al. (8) reported a MAR index of 0.22 in heat-resistant *V. parahaemolyticus* isolates from environmental seafood samples, thereby correlating vibriosis persistence in the US with *V. parahaemolyticus* multiple hardy properties (i.e., heat-resistance and MAR).

The detection protocol of BAM, including gene-specific DNA analysis (i.e., *tdh*, *trh*, and *tlh*) and one-step enrichment-based culture, are routinely in use to investigate total *V. parahaemolyticus* (9). The two-step enrichment method of Meza et al. (8) employs APW enrichment of sample containing presumptive *V. parahaemolyticus* followed either by direct culture on TCBS agar

plates (i.e., BAM's one-step enrichment method) (9) or heat-treatment then culture on TCBS agar plates (i.e., two-step enrichment method) and exclusively recovered VBNC, heat-resistant (i.e., 80 °C, 20 min) *V. parahaemolyticus* isolates (i.e., VHT1 and VHT2) along with regular *V. parahaemolyticus* (i.e., isolated with no 80 °C treatment), respectively.

Meza et al. (8) findings suggest that total *V. parahaemolyticus* (i.e., heat-resistant and regular forms) can be recovered simultaneously using one of the two-steps of their modified enrichment method, that the heat-resistant group could withstand pasteurization temperatures (i.e., 65 °C, 8 h), and that further investigation using large sample sizes and sources are worthwhile for food safety sake. Their findings further implicate that the existing detection and cooking protocol of FDA (9) and USDA (14), respectively, are ineffective in tackling this form of *V. parahaemolyticus* (i.e., heat-dependent resuscitated, pasteurization-resistant strains), thereby rendering a public health threat.

The present study aims to evaluate retail shellfish seafood for heat/pasteurization-resistant *V. parahaemolyticus* and implicate the root cause of persistent vibriosis in light of the food safety guideline availability for detection (i.e., BAM) (9), processing, and cooking (i.e., USDA) (14). To our knowledge, this is the first investigation of retail seafood for heat/pasteurization-resistant *V. parahaemolyticus* using the modified two-step enrichment method of Meza et al. (8). The study evaluated locally wild-caught and non-locally farm-raised retail seafood samples available domestically for dictating the prevalence and distribution of heat/pasteurization-resistant *V. parahaemolyticus* pathogen in the US.

2. MATERIALS AND METHODS

2.1 Sample collection. In November 2019, the test samples (i.e., retail shellfish samples), including raw, deveined/un-deveined, shell-on, tail-on, easy-peel, headless shrimp, crab, and oysters, both wild-caught and farm-raised, were separately collected from the local grocery (i.e., Best Aquaculture Practices, BAP, certified; 11/10/19-11/23/19) and a non-local retail (i.e., Fulton Fish Market; ordered on 11/23/19) markets in frozen conditions each in two or three replicates and kept frozen until experimental processes (Table 1).

Table 1: Sample information

Seafood Species	Retail source	Condition	Raised origin	Certification	Sample ID
Gulf Crab	Walmart	Raw, frozen	Wild	None	GC
Fremont Fish Market Shrimp	Aldi	Raw, frozen	Farm-raised	BAP	FF
Great Value Shrimp	Walmart	Raw, frozen	Farm-raised	BAP	GF
Gulf shrimp	Walmart	Raw, frozen	Wild	None	GS
Atlantic Oysters	Fulton Fish Market	Raw, frozen	Wild	None	AW
Pacific Oysters	Fulton Fish Market	Raw, frozen	Farm	None	PF
Pacific Oysters	Fulton Fish Market	Raw, frozen	Wild	None	PW

2.2 Bacterial enrichment. The modified two-step enrichment method of Meza et al. (2022) was adapted for isolating presumptive *V. parahaemolyticus* (Figure 1). Briefly, samples were thawed at room temperature and immediately processed the same day. Sample solutions (250 g, *wt/v*) containing 25 g samples and buffered peptone water (pH 8.6) supplemented with 8% NaCl were homogenized in a lab Stomacher® 400 Circulator lab blender (Seward, Weber Scientific, Hamilton, NJ, USA), incubated at 35 °C for 48 h, and homogenate solutions were heated (80 °C, 20 min) prior to serial dilutions plating on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar media (TCBS, Difco, Detroit, MI, USA) or the homogenate serial dilutions (i.e., with no pre-heated step) were directly plated on TCBS agar media followed by plate incubation at 35 °C until heat-resistant and regular presumptive *V. parahaemolyticus* colonies emerged, respectively.

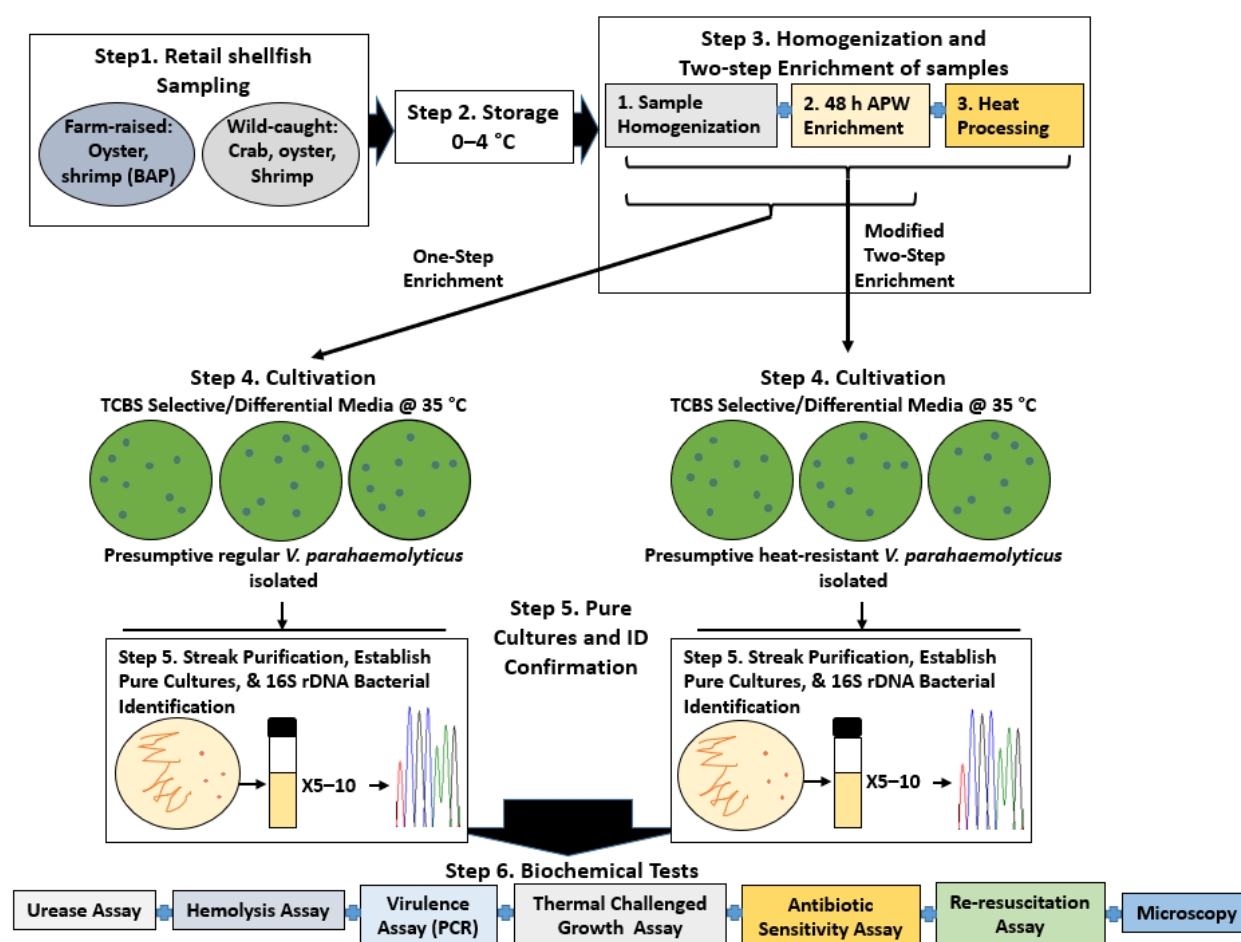


Figure 1: Retail *V. parahaemolyticus* experimental study chart adapted from Meza et al. (8).

Step 1, sample collection; step 2, storage of samples before experimental processes; step 3, isolation of pure presumptive *V. parahaemolyticus* cultures; step 4, *V. parahaemolyticus* confirmation using 16S rRNA bacterial identification; step 5, biochemical characterization of confirmed *V. parahaemolyticus*.

2.3 Isolates culture and storage conditions. As described by Meza et al. (8), bacterial colonies were streaked for purification on Brain Heart Infusion agar (BHI, Difco, Detroit, MI, USA) supplemented with 3% NaCl, and pure cultures were suspended in sterile BHI broth supplemented with 10% sterile glycerol before storage in a -70 °C freezer. Thawed cultures, including confirmed *V. parahaemolyticus* (Table 2), were inoculated (1/100, v/v) into sterile fresh BHI broth supplemented with 3% NaCl, incubated at 35 °C for 16-20 h, and sub-cultured in fresh sterile BHI broth another time before fresh culture experiments.

Table 2: *V. parahaemolyticus* isolates

Strain ID	Isolation condition	Source
VHT51	Non-heated	AW-1
VHT52	Non-heated	AW-1
VHT54	Non-heated	PF-1
VHT55	Non-heated	PW-1
VHT56	Non-heated	PW-1
VHT71	Non-heated	GF-1
VHT73	Non-heated	GF-1
VHT77	Heated	AW-2
VHT78	Heated	AW-2
VHT1	Heated	(8)
VHT2	Heated	(8)

2.4 Bacterial identification.

2.4.1 Genome extraction. Bacterial genomes were extracted using the bead-collision method of Tiong and Muriana (15). Briefly, fresh subculture pellets (12K RPM, 2 min) (VWR, Suwanee, GA, USA) were washed twice by centrifugation (12K RPM, 2 min) and resuspension in fresh sterile water. After final centrifugation, cell pellets were suspended in 0.1 mL sterile Tris buffer (10 mM, pH 7.4) followed by physical lysis with sterile glass beads (5 µm), and the cytoplasmic DNA supernatants were collected after centrifugation (12K RPM, 1 min) and stored at -20 °C before experimental analyses. The DNA was qualitative- and quantitatively assessed using a UV spectrophotometer (Thermo Scientific, South San Francisco, CA, USA).

2.4.2 PCR. PCR mixtures were formulated in accordance with the GoTaq Flexi DNA Polymerase's instructions (Promega, Madison, WI, USA). Each reaction mixture containing 5× PCR buffer (Promega), 0.4 µM of gene-specific primers (Table 3) (IDT, Coralville, IA, USA), 1.5 mM MgCl₂ (Promega), 0.2 mM deoxynucleoside triphosphate mix (dNTPs, Fisher Scientific, Fair Lawn, NJ, USA), <0.5µg/50µl template DNA, and 1.25 U of GoTaq polymerase (Promega) was reacted in a GeneAmpPCR System 9700 Thermal Cycler (Applied Biosystems, Thermo Scientific) following PCR cycle step 1, 1 cycle of 5 min denaturation at 95 °C; step 2, 40 cycles of 1 min denaturation at 95 °C, 40 s primer-dependent annealing (Table 3), DNA extension at 72 °C (amplicon-size-dependent incubation time); step 3, 1 cycle of extended DNA extension for 10 min at 70 °C before infinite holding at 4 °C.

2.4.3 Gel electrophoresis. PCR amplicon-specific sizes (bp) were analyzed in an agarose gel (1.6% agarose + 1 x Tris-borate-EDTA buffer) containing GelStar™ Nucleic Acid Gel Stain solution (ratio 5 µL stain solution: 50 mL gel solution, Lonza Walkersville Inc., Walkersville, MD, USA) using a UV transilluminator (Ultra-Lum UV Transilluminator Muvb-20).

Table 3: Primer pairs and conditions used in this study

Gene ID	Primer Sequence	Amplicon Size (bp)	Melting Temp. (°C)	Annealing Temp. (°C)	Source
16S-515	F- GTGCCAG CMGCCG CGGTAA	900	65.2	56	16
16S-1391	R- GACGGG CGGTGTG TRCA		59.8	56	16
<i>tdh</i>	F- GTARAGG TCTCTGA CTTTTGG AC	229	66	56	17
	R- CTACAGA ATYATAG GAATGTT GAAG		66		
<i>tlh</i>	F- AAAGCG GATTATG CAGAAG CACTG	450	70 68	56	17
	R-				

	GCTACTT				
	TCTAGCA				
	TTTTCTC				
	TGC				
	F-				
	CCATCM				
	ATACCTT				
	TTCCTTC				
<i>trh</i>	TCC	207	66	59	17
			60		
	R-				
	ACYGTCA				
	TATAGGC				
	GCTTAAC				
	F-				
	CTAACTT				
	TGAACAA				
	CAAATC				
<i>ure</i>	TCGC	5.6k	53.3	55	18
			52.8		
	R-				
	GATCTCC				
	CTTTATT				
	TTTATGT				
	CGGA				

2.5 Urease assay. *V. parahaemolyticus* urease activity was investigated as described by the kit testing instructions. Briefly, a urea test reaction containing freshly sub-cultured bacterial cells re-suspended in 1 mL of peptone solution (0.1%) supplemented with a urea pellet (Hardy Diagnostics, Santa Maria, CA, USA) was incubated at 35 °C for 24 h, or until a visible pink solution (i.e., positive urease test) developed.

2.6 Hemolysis assay.

2.6.1 Blood agar preparation. Sheep and human (i.e., from a > 50-year-old male donor) erythrocytes were acquired commercially. BHI agar (pH 7.4) supplemented with NaCl (1%) and

sheep blood (5%, v/v) (19) or human erythrocytes (~2%, v/v) (20) were made as described previously.

2.6.2 Blood agar inoculation. Spotting inoculation of Xu et al. (19) was adapted to examine bacterial hemolytic activity. Subcultures of *V. parahaemolyticus* in BHI culture broth were distantly spotted on blood agar plates, and inoculated plates were incubated at 35 °C for 24 h before hemolysis examination. The hemolysis-positive *V. parahaemolyticus* (i.e., VHT2) of Meza et al. (8) was employed in the assay (i.e., BHI agar containing human erythrocytes) for beta-hemolysis confirmation (21). For streaking inoculation (22, 23), subculture linear/zig-zag streaks on blood agar plates were generated using a sterile inoculating loop, and inoculated plates were incubated at 35 °C for 24 h before hemolysis examination.

2.7 Thermal sensitivity test. The heat-challenged viability assay of Meza et al. (8) was adapted for examining VBNC-reverted *V. parahaemolyticus*. Screw cap tubes containing sterile fresh culture broth were inoculated with thawed select *V. parahaemolyticus* strains (VHT51, VHT52, VHT77, VHT78) (1/100) and incubated for 20-48 h at 35 °C. The tubes containing replenished cultures (i.e., no visible growth) were subsequently incubated in a water bath (63 °C, 7 h or 80 °C, 20 min) followed by incubation (35 °C) until colony emerged (i.e., 72 h) and viable colony enumeration or refrigeration (i.e., 4 °C), incubation until colony emerged (i.e., 72 h) and viable colony enumeration.

2.8 Antibiotic sensitivity assay. To assay bacterial sensitivity to antibiotics, including chloramphenicol (C30/Chl30), ciprofloxacin (CIP5), erythromycin (E15/Ery15), Gentamicin (GM10/Gen10), nalidixic acid (Na30/Nal30), Neomycin (N30/Neo30), penicillin (P10/Pen10), streptomycin (S10/Str10), and tetracycline (Te30/Tet30), a disk diffusion method was conducted as described by Tan et al. (24). Each commercial disc (Becton Dickinson BBL™Sensi-Disc™, San Jose, CA, United States) consisted of an antibiotic concentration measured in microgram (µg/disc). The Clinical and Laboratory Standards Institute (CLSI) Document M100-S21 (M2): Disk Diffusion Supplemental Tables, Performance Standards for Antimicrobial Susceptibility Testing, and the zone diameter interpretative guidelines were adapted to conduct tests and dictate bacterial sensitivity to antibiotics tested, respectively (25). The multiple antibiotic resistance (MAR) index was determined according to Meza et al. (8) (i.e., MDR # / total antibiotics tested).

2.9 Statistical analysis. Using one-way analysis of variance (ANOVA), the statistical significance for bacterial antibiotic inhibition zones among retail *V. parahaemolyticus* was determined to articulate the diversity of the pathogen isolates among the heat-resistant group, regular group, and between the heat-resistant group and regular group. $P < 0.05$ was marked as the significant difference for retail *V. parahaemolyticus* inhibition zones compared (in this study).

2.10 Re-resuscitation assay. VBNC-reverted *V. parahaemolyticus* isolates were re-resuscitated according to the modified two-step enrichment technique of Meza et al. (8). Briefly, thawed cultures in BHI supplemented with 10% glycerol were inoculated (1/10, v/v) into BPW (5%, pH 8.6) supplemented with 8% NaCl and incubated at 35 °C for 48 h. After enrichment incubation, the cells were plated (i.e., step one of the technique) or heated, followed by refrigeration (4 °C, >8 hr) and plating (i.e., steps one and two of the technique) on TCBS agar, and inoculated TCBS agar plates were incubated at 35 °C until colonies emerged. Pure cultures (if applicable) were established, and subcultures were made prior to experimental applications as aforementioned (in this study).

2.11 Light Microscopy. Non-viable *V. parahaemolyticus* isolates, both regular (i.e., VHT51, VHT52) and heat-resistant strains (i.e., VHT77, VHT78), post-re-resuscitation step were smeared on microscope slides using a sterile inoculating loop and Gram-stained, as described previously for Gram-negative bacteria staining (26). Stained cell smears were examined under a light microscope (LabomedCxL LED) with a magnification (1,000 X) that created quality images, and the cellular morphology was recorded.

3. RESULTS

3.1 *V. parahaemolyticus* prevalence. A total of 17 retail shellfish samples were evaluated for the prevalence of both 48 h enrichment regular and 48 h enrichment followed by thermal-treated *V. parahaemolyticus* isolates (Figure 1). *V. parahaemolyticus* contaminant was detected in the samples tested (5/17; 29.4%), with the majority were regular *V. parahaemolyticus* carriers (78%) of oyster samples (4/17; 23.5%) (Table 1). It is worth noting that oyster shellfish was the leading carrier of the pathogen (i.e., 4 of 5 *V. parahaemolyticus*-positive samples) (Table 4). Of the positive samples, *V. parahaemolyticus* was more prevalent in wild-caught shellfish (i.e., 3/5 *V. parahaemolyticus*-positive samples) than the counterpart (i.e., farm-raised shellfish) (Table 4). The regular or heat-resistant isolates (i.e., detected in 20% of the *V. parahaemolyticus*-positive samples) (Figure 2) were exclusively detected in one of the oyster samples tested (Table 2, Table 4), and the latter group was exclusively detected in wild-caught oyster analyzed (Table 2, Table 4).

Table 4: Shellfish seafood sources and the *V. parahaemolyticus* carriers

Species	Source	Sample size ^a	Raised origin	Sample ID	VP detected (+ / -) ^b
Crab	Gulf	3	Wild	GC-1, GC-2, GC-3	- - -
Oyster	Atlantic	2	Wild	AW-1, AW-2	+ +
Oyster	Pacific	2	Wild	PW-1, PW2	+ -
Oyster	Pacific	2	Farm	PF-1, PF-2	+ -
Shrimp	Fremont Fish Market	3	Farm	FF-1, FF-2, FF-3	- - -
Shrimp	Great Value	3	Farm	GF-1, GF-2, GF-3	+ - -
Shrimp	Gulf	2	Wild	GS-1, GS-2	- -

^a Each size represents an individually packaged sample.

^b Number of + or – indicates the number of *V. parahaemolyticus* (VP) positive or negative samples.



Figure 2: Heat-resistant *V. parahaemolyticus* VHT77 (A) and VHT78 (B) isolates emerged on TCBS agar after the heated processing (i.e., 80 °C, 20 min) of the modified two-step enrichment method of Meza et al. (8).

3.2 Thermal viability of heat-resistant *V. parahaemolyticus*. *V. parahaemolyticus* isolates resuscitated by enrichment and subsequent heat treatment (80 °C, 20 min) (i.e., heat-resistant *V. parahaemolyticus*) were re-evaluated for their thermal-challenged viability. Using VBNC-reverted cultures (VHT51, VHT52, VHT77, VHT78), both 5 min and 20 min thermal treatment at 80 °C did not exhibit any viable *V. parahaemolyticus* on agar plates (Table 5). Subsequent

prolonged thermal evaluation of these isolates (i.e., both regular and heat-resistant, VBNC-reverted isolates) using a reduced, pasteurization temperature (i.e., 63 °C, 7 h) followed by viable assessment after 72 h incubation at 35 °C did not exhibit any visible viability (Table 5).

3.3 Virulence phenotype characterizations. *V. parahaemolyticus* hemolytic phenotypes were evaluated for virulence indication. Differential Kanagawa positive hemolytic activities (i.e., on BHI supplemented with human blood) (Table 5, Figure 3) were detected in regular *V. parahaemolyticus* (VHT54, VHT55, VHT56) post 48 h incubation at 35 °C as opposed to no-visible hemolytic activity exhibited on BHI agar supplemented with sheep blood (Figure 4). However, differential colony morphologies of the isolates were visually distinctive among isolates tested on the latter media (i.e., sheep blood-supplemented blood agar) (Figure 4).

Heat-resistant VHT77 and VHT78 isolates did not exhibit any visible hemolytic activity on BHI supplemented with sheep blood (i.e., VHT78, hemolytic negative; VHT77, did not exhibit visible growth) (Figure 4) or human blood (i.e., did not exhibit visible growth) (data not shown). They exhibited diverse growth behaviors (i.e., colony morphology or +/- viability) between themselves (i.e., VHT77 vs. VHT78) and among the regular isolates (i.e., VHT52, VHT54, VHT55, VHT56, VHT71) when cultured on BHI agar supplemented with sheep blood (Figure 4). Subsequent urease examination revealed urease-positive *V. parahaemolyticus* in the isolates tested (Table 5, Figure 5).

Table 5: Phenotypes of select *V. parahaemolyticus* isolates

Strain ID	Urease	Sheep blood hemolysis	Human blood hemolysis	Thermal-challenged viability ^b			
				80 °C	63 °C	80 °C + 4 °C	63 °C + 4 °C
VHT51	ND ^a	ND ^a	ND ^a	x	x	x	x
VHT52	+	-	ND ^a	x	x	x	x
VHT54	+	-	+	ND	ND	ND	ND
VHT55	+	-	+	ND	ND	ND	ND
VHT56	+	-	+	ND	ND	ND	ND
VHT71	ND ^a	-	ND ^a	ND	ND	ND	ND
VHT73	ND ^a	ND ^a	ND ^a	ND	ND	ND	ND
VHT77	+	ND ^a	ND ^a	x	x	x	x
VHT78	ND ^a	-	ND ^a	x	x	x	x

^a Not determined; partially due to the experimental supply limitations and bacteria (VHT77, VHT78) reentering into a VBNC stage (i.e., VBNC-reverted bacteria). ^bThe isolates of VBNC-reverted *V. parahaemolyticus* (i.e., no visible growth turbidity at post-cultivation of thawed cultures in fresh BHI)

were used for determining their thermal viability phenotypes, including a pasteurization temperature (i.e., 63 °C).

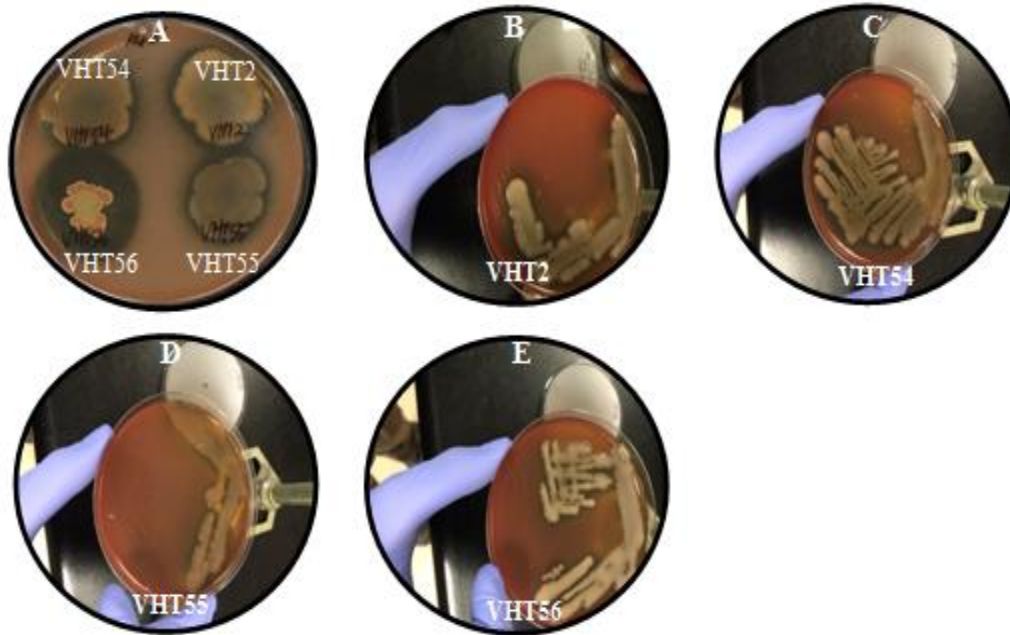


Figure 3: Kanagawa phenomenon at post-incubation (35 °C, 24 h) of select retail *V. parahaemolyticus* isolates spotted (A) or streaked (B-E) on BHI supplemented with human erythrocytes. Each image represents one of three test replicates.



Figure 4: Colony morphology of regular (VHT52, VHT54, VHT55, VHT56, VHT71) and heat-resistant (VHT77, VHT78) *V. parahaemolyticus* isolates at post-cultivation (35 °C, 48 h) on BHI agar supplemented with sheep blood. The image represents one of three test replicates.

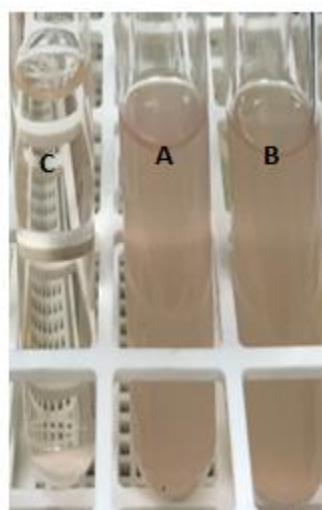


Figure 5: Urease availability (i.e., 35 °C, 24 h) of retail *V. parahaemolyticus* VHT52 (A) and VHT55 (B) isolates compared with a negative control (c) tube containing urea solution and urease-negative culture. The image represents one of three experimental replicates.

3.4 Virulence genotype characterizations. To correlate *V. parahaemolyticus* genotypes with the hemolysin and urease phenotypes, select genomes were analyzed with gene-specific PCR for hemolysin and urease genotypes (Table 3). All isolates tested positive for *tlh*, and none of them exhibited the amplicons for other hemolysin genes tested (i.e., *tdh* and *trh*) (Table 6, Figure 6). Subsequent urease gene examination (Table 3) revealed non-detectable amplicons, thereby indicating urease-negative *V. parahaemolyticus* in all isolates, including the pasteurization-resistant VHT1 and VHT2 isolates of Meza et al. (8) (Table 6, Figure 6).

Table 6: Virulence genes possessed in heat-resistant *V. parahaemolyticus* isolates

Strain ID	<i>tdh</i>	<i>trh</i>	<i>tlh</i>	<i>ure</i>
VHT51	ND ^a	-	ND ^a	-
VHT52	-	-	+	-
VHT54	-	-	+	-
VHT55	-	-	+	-
VHT56	-	-	+	-
VHT71	-	-	+	-
VHT73	-	-	+	-
VHT77	-	-	+	-
VHT78	-	-	+	-
VHT1 ^b	-	-	+	-

VHT2 ^b	-	-	+	-
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^a Notdetermined.

^b Heat-resistant *V. parahaemolyticus* isolates of Meza et al. (8).

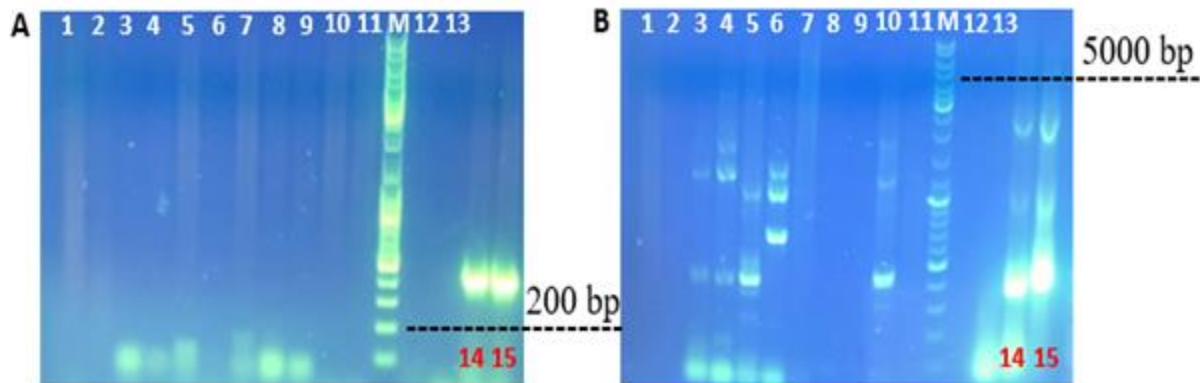


Figure 6: PCR amplicons of *tdh*⁻ (A) and *ure*⁻ (B) *V. parahaemolyticus* isolates this study and pasteurization-resistant VHT1 and VHT2 of Meza et al. (8) visualized on a UV light box after electrophorized on 1.6% agarose gel pre-stained with GelStar® Nucleic Acid Stain. Lanes 1, VHT1; 2, VHT2; 3, HT51; 4, VHT52; 5, VHT54; 6, VHT55; 7, VHT56; 8, VHT71; 9, VHT73; 10, VHT77; 11, VHT78; M, 1Kb DNA ladder; 12, - PCR control (*trh* primers; no bacterial genomes added); 13, - PCR control (*tlh* primers; no bacterial genomes added); + PCR control (VHT71 genome); + PCR control (VHT77 genome).

3.5 Antibiotic sensitivity profile. Retail isolates of *V. parahaemolyticus* were evaluated for their antibiotic susceptibility profiles. Mixed antibiotic susceptibility profiles (Figure 7) and MAR (Table 7) were detected in retail isolates of *V. parahaemolyticus*. Significant antibiotic susceptibility-variant *V. parahaemolyticus* were detected for Ch30, Ci5, Er15, Ge10, Na30, and Te30 antibiotics ($P < 0.05$) (Figure 7). The isolates consisted of *V. parahaemolyticus* with 0 (VHT54, VHT55), 0.22 (VHT78), and 0.44 (VHT52, VHT56, VHT71) MAR indexes, and the greater MAR strains were more prevalent in the wild-caught isolates compared with the farm-raised isolates (i.e., 2 out of 3) (Table 7).

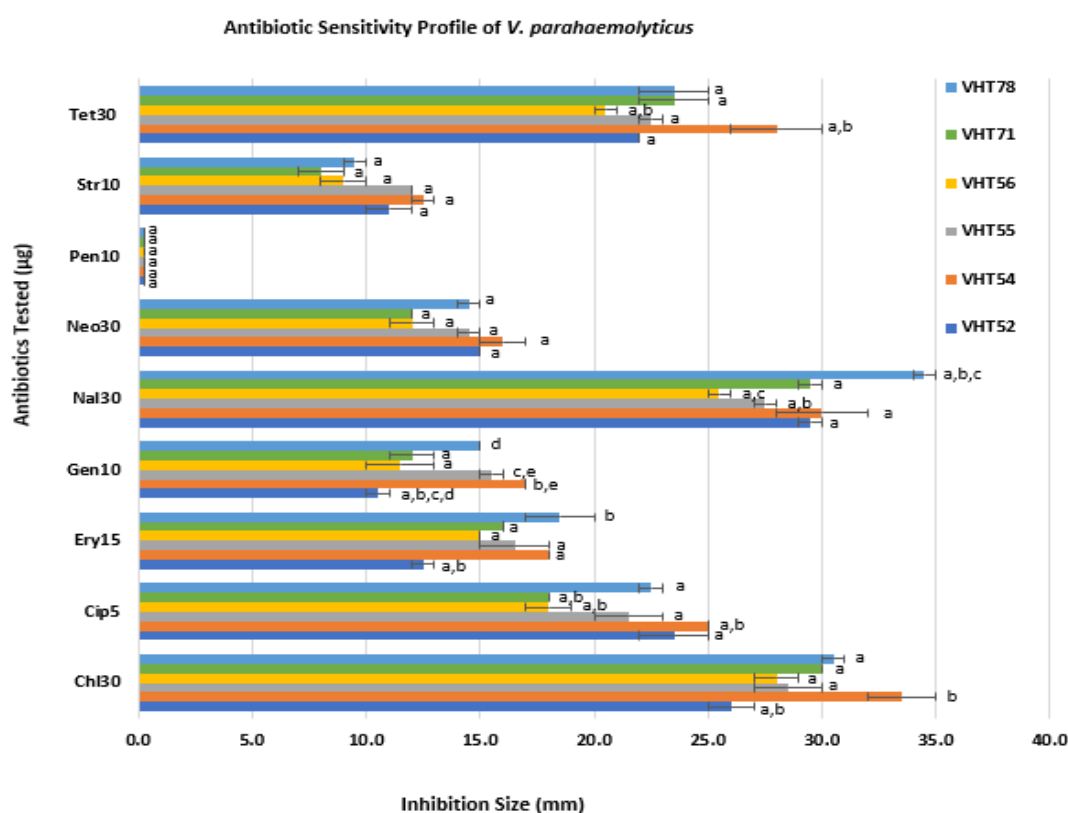


Figure 7: Antibiotic sensitivity profile of *V. parahaemolyticus* isolates. Significant variation in isolates' sensitivity to each antibiotic is dictated by increasing letter in the next strain; a similar letter is used among no significant variation isolates.

Table 7: Antibiotic Profile interpretation^a and the multiple antibiotic resistance (MAR) of select retail *V. parahaemolyticus* isolates

Strain ID	C3 0	CIP 5	E1 5	GM 10	NA 30	N30	P1 0	S1 0	Te 30	MAR #	MAR index
VHT											
52	S	S	R	R	S	I	R	R	S	3	0.44
VHT											
54	S	S	I	S	S	I	R	I	S	0	0.11
VHT											
55	S	S	I	S	S	I	R	I	S	0	0.11
VHT											
56	S	I	I	R	S	R	R	R	S	3	0.44
VHT											
	S	I	I	R	S	R	R	R	S	3	0.44

71												
VHT												
78	S	S	I	S	S	I	R	R	S	I		0.22

^aThe antibiotic sensitivity interpretation is dictated according to the Clinical and Laboratory Standards Institute (CLSI) M45 [47] and M100-S21(M2) (i.e., found in the antibiotic product user manual) zone diameter (mm) interpretative guidelines for *Vibrio* species and Enterobacteriaceae/enterococci/*Escherichia coli*, respectively. S, susceptible; I, intermediate; R, resistant. NA, not applicable.

3.6 Re-resuscitation profile. All VBNC-reverted *V. parahaemolyticus* isolates tested (Table 8), including the regular (i.e., VHT51, VHT52) and heat-resistant (i.e., VHT77, VHT78) strains, were re-resuscitated using the same modified two-step enrichment technique of Meza et al. (8) as it was used for isolating *V. parahaemolyticus* from seafood samples. Re-resuscitation of VBNC-reverted *V. parahaemolyticus*, including enrichment only (i.e., step 1), enrichment + heat selection (i.e., steps 1 and 2), and enrichment + heat selection + refrigeration (4 °C) (i.e., steps 1 and 2), did not exhibit any viable colonies on TCBS agar plates (Table 8).

Table 8: Re-resuscitation and Gram-staining microscopy of VBNC-regenerated *V. parahaemolyticus* isolates

Isolate	One-step ^a	two-step ^a	BHI ^a	Cell intact structure	Cell morphology
VHT1	NA	NA	x	NA	NA
VHT2	NA	NA	x	NA	NA
VHT51	x	x	x	√	Oval/round
VHT52	x	x	x	√	Oval/round
VHT54	NA	NA	x	NA	NA
VHT55	NA	NA	x	√	Oval/round
VHT56	NA	NA	x	√	Oval/round
VHT71	NA	NA	x	NA	NA
VHT73	NA	NA	x	NA	NA
VHT77	x	x	x	√	Oval/round
VHT78	x	x	x	√	Oval/round

^a Incubation conditions before examination. One-step, cells incubated in BPW for 48 h at 35 °C; two-step, one-step incubated cells were heated at 80 °C for 20 min followed by direct plating or refrigeration prior to plating. NA, not applicable; x, non-culturable per visible turbidity examination; √, yes.

3.7 Microscopy. Subsequently, the viability of VBNC-reverted *V. parahaemolyticus* isolates (i.e., VHT51, VHT52, VHT55, VHT56, VHT77, VHT78) in enrichment alone (i.e., BPW, one-step) (i.e., VHT51, VHT52, VHT77, VHT78), enrichment + heat selection (i.e., the modified two-step enrichment method) (i.e., VHT51, VHT52, VHT77, VHT78), or sub-culturing medium (i.e., inoculated and incubated BHI at 35 °C for 48h) (i.e., VHT51, VHT52, VHT55, VHT56, VHT77, VHT78) were analyzed for intact cells using Gram Staining microscopy. Stained cells exhibited non-rod-shaped, intact cells (Table 8).

4. DISCUSSION

Previous work exclusively recovered heat-resistant environmental *V. parahaemolyticus* using the modified two-step enrichment technique's heating process of Meza et al. (2022) which was then validated to be the pasteurization resistant isolates (i.e., 65 °C, 7 h). Using this technique, retail seafoods exhibited 29 presumptive *V. parahaemolyticus* and, of all, nine (i.e., 31%) (Table 2) isolates were confirmed *V. parahaemolyticus*, including the regular (7/9, 78%) and heat-resistant (2/9, 22%) forms, thereby confirming the heat-resistant *V. parahaemolyticus* prevalence (i.e., relatively low) of Meza et al. (2022). Retail seafoods that were validated to be *V. parahaemolyticus* carriers (i.e., 5 carriers of 17 samples tested) were primarily wild-caught (i.e., 3 carriers of 9 wild-caught samples tested, 33%) as opposed to farm-raised (i.e., 2 carriers of 8 farm-raised samples tested, 25%) (Table 4). This could be attributed to the efficient manageability (i.e., antibiotic treatment) (13,27) of seafood-raising farms as opposed to wild free-moving seafood organisms. The exclusive non-availability of heat-resistant *V. parahaemolyticus* in farm-raised seafoods (in this study) (Table 1, Table 2) could be attributed to global warming-elicited gene transfer among the pathogen and its heat-resistant co-inhabitants in the wild, open ecosystem (i.e., ocean) as described previously by Shishir et al. (28) and Vezzulli et al. (29) (Table 2, Table 4). Interestingly, one of the wild-caught sample duplicates (i.e., Atlantic wild oyster x 2 samples) each exclusively exhibited either the regular (i.e., sample AW-1 exhibited VHT51, VHT52) or heat-resistant isolates (i.e., sample AW-2, VHT77, VHT78) (Table 2). This indicates that the existing detection enrichment protocol of BAM (9) will fail food safety from this evolved strain of *V. parahaemolyticus* (i.e., heat-dependent resuscitation of VBNC *V. parahaemolyticus*), hence, an improved testing protocol is warranted, and that mixtures of regular and heat-resistant *V. parahaemolyticus* could coexist in one seafood origin. In addition, *V. parahaemolyticus* contaminant was more prevalent in oysters than other retailed seafood organisms tested (Table 4), thereby, agreeing with previous reports (8,30,31). The prevalence of *V. parahaemolyticus*, including the regular, *tlh*hemolysin-positive VHT71 and VHT73 (Table 2), in one of the BAP-restricted retail seafood sources (GF-1) (Table 1, Table 2) could be partially due to their ineffective cleaning to completely remove the adherent bacterial contaminant, including *V. parahaemolyticus* (32,33), and failed inspection protocol.

The retail heat-resistant isolates were unable to remain viable at post-reheating (80 °C) or pasteurization conditions (i.e., 63 °C, 7 h) (Table 5). The sensitivity of VHT77 and VHT78 (i.e., heat-resistant *V. parahaemolyticus*) to heat-resistant *V. parahaemolyticus* isolation temperature of 80 °C were equally observed in the VHT1 and VHT2 isolates of Meza et al. (2022). The non-viability of VBNC-reverted VHT77 and VHT78 isolates to prolonged pasteurization process (i.e., 63 °C) (Table 5) is inconsistent with pasteurization-resistant VHT1 and VHT2 *V. parahaemolyticus* isolates of Meza et al. (2022). This could be attributed to the availability of diverse groups of heat-resistant *V. parahaemolyticus* (i.e., regular/non-thermal resistant, heat resistant/non-pasteurization resistant, and heat/pasteurization-resistant) or the fact that viable and culturable status is a pasteurization resistance prerequisite via the chaperone-like activity of DnaI (34), DnaK (35), GroEL, and GroES (34,35) proteins as evident in a surviving Gram-negative bacterium to heat stress (36). These findings (i.e., in this study and Meza et al., 2022), it's worth noting that none of the regular isolates (in this study) were viable to all thermal tests studied (Table 5), suggesting the presence of three physically distinct groups of *V. parahaemolyticus* isolates (i.e., regular form isolated using BPW enrichment vs pasteurization-resistant form isolated using the Meza's modified two-step enrichment method (8) vs non-pasteurization-resistant form isolated using the Meza's modified two-step enrichment method).

Heat-resistant *V. parahaemolyticus* diversity was further noted in their phenotypical and genotypical properties. Regular, hemolytic *V. parahaemolyticus* was discovered in one of the BAP-restricted retail seafood sources (i.e., VHT56) (Table 1, Table 4), thereby suggesting the ineffectiveness of the existing detection or processing protocol. The heat-resistant isolates of VHT77 and VHT78 exhibited a dormant stage (i.e., VBNC-reverted) in most biochemical tests, attributed to cold storage conditions described by Mizunoe et al. (2), Yang et al. (37), and Di Salvo et al. (31). The Kanagawa hemolytic test (Figure 3) revealed that spotted *V. parahaemolyticus* could exhibit clearer hemolysis on the blood agar than the streaked cultures, thereby recommending using this technique for conducting *V. parahaemolyticus* hemolysis study. Further, the hemolysis test (i.e., using human erythrocyte-supplemented blood agars) produced consistent hemolysis activity in a hemolysis-positive isolate (i.e., VHT2) as reported by Meza et al. (8), and, hence, suggests the need to employ human erythrocytes in *V. parahaemolyticus* Kanagawa phenomenon assay as opposed to using sheep blood in the assay described by Xu et al. (19), and the prevalence of hemolysis-positive-implicated *V. parahaemolyticus* pathogenicity as described by Wang et al. (10) in retail seafood tested (in this study) (Figure 3, Table 5). Okuda et al. (38) and Park et al. (39) reported a positive correlation between the hemolysin Trh/trh and Ure/ure phenotypes/genotypes. This relationship (i.e., hemolysis and urease positive phenotypes) was evident in retail, regular (i.e., VHT54, VHT55, VHT56) (in this study) and Meza et al. (8) environmental, heat-resistant (i.e., VHT2) *V. parahaemolyticus* isolates (Figure 3, Figure 5, Table 5). Further, Takahashi et al. (40) and Wang

et al. (10) separately implied *V. parahaemolyticus* Kanagawa phenomenon and *tdh/trh* inconsistent positive correlation, suggesting the need for studying both their phenotypes and genotypes to improve the accuracy of bacterial virulence characterization results.

V. parahaemolyticus pathogenicity-implicated gene-specific PCR, including *tdh*, *trh*, *tlh*, and *ure* (Table 6), were conducted to dictate/correlate with the determined phenotypes of *V. parahaemolyticus* isolates (in this study and Meza et al.) (Table 5) (8), including the VBNC-reverted, regular, heat-resistant, and pasteurization-resistant isolates (Table 5, Table 6). The genetic examinations, however, did not correlate with the isolates' phenotypes (i.e., *trh*⁻ and urease positive activity, *ure*⁻ and urease positive activity) determined in this study (Table 5, Table 6). Taking together (in this study, 8,10,38,39,40), these inconsistent observations, including hemolysis-positive *tdh*⁻/*trh*⁻/*tlh*⁺ (in this study) (Table 5, Table 6) as opposed to Takahashi et al. (40) and Wang et al. (10) hemolysis-negative *tdh*⁺/*trh*⁺ strains, and urease-positive (Table 5, Table 6) in *ure*⁻ and *trh*⁻ isolates tested (in this study), suggest the availability of a functional, gene sequence-manipulated protein (i.e., urease-positive vs *ure*⁻, in this study) that compromises PCR annealing and amplification by primers, thereby, rendering PCR detection failures and non-correlated phenotypes and genotypes (i.e., urease-positive vs *ure*, *ure* and *trh* correlation), as Okuda et al. (i.e., *trh* gene variants) (38) and Tiong and Muriana noted previously (15), and hemolysis-positive retail *V. parahaemolyticus* possessing detectable *tlh* and other functional hemolysins described previously (35,41).

Wong et al. (11) revealed quinolones (i.e., unspecified), cephalosporins, tetracyclines, and penicillins as the frequently-prescribed antibiotics in clinical therapy for vibriosis (i.e., including *V. parahaemolyticus* infections) and cholera while Sharma et al. (13) indicated quinolones (i.e., including ciprofloxacin and non-nalidixic acid quinolone antibiotics) and tetracyclines are among the frequently used antibiotics in seafood farming. The present assessment of *V. parahaemolyticus* sensitivity to these antibiotics (i.e., quinolones, ciprofloxacin, nalidixic acid, tetracyclines) revealed no antibiotic resistance concern (Table 7). However, increasing resistance to ciprofloxacin was evident in a subset of the isolates (i.e., intermediate sensitivity in VHT56 and VHT71) (Table 7). The heat-resistant *V. parahaemolyticus* VHT78 of wild-caught retail seafood posed a consistent antibiotic resistance profile with the VHT1 and VHT2 (i.e., penicillin and streptomycin resistances), environmental isolates, of Meza et al. (8), confirming the availability of heat-resistant *V. parahaemolyticus* with penicillin and streptomycin resistances (Table 7). The highest MAR *V. parahaemolyticus* of VHT52, VHT56, and VHT71 (Table 7) were isolates from the farm (VHT71) and wild-caught (VHT52 and VHT56) seafoods (Table 2), indicating potential antibiotic cross-contamination of the two environments via human activities, including antibiotic-treated biological disposals and the availability of antibiotic-retaining organic and microplastic wastes (12,42). These isolates' resistance to ampicillin equivalent

antibiotic (i.e., penicillin) (Table 7) conforms to the high prevalence of penicillin-resistant-*V. parahaemolyticus* (8,12) and their natural resistance to the antibiotic, as described previously (42).

Mizunoe et al. (2), Yang et al. (37), and Di Salvo et al. (31) indicated the availability of VBNC *V. parahaemolyticus* in the lab strains (2) and retail seafood (31,37) upon cold temperature exposures (4 °C – -25 °C), suggesting the reversibility of resuscitated *V. parahaemolyticus* lab strains (i.e., VBNC-reverted strains) into the VBNC stage during frozen storage of the isolates in this study (i.e., found in both regular and heat-resistant isolates) (Table 8). It's worth noting that the present study discovered, for the first time, a VBNC-reverted form in heat-resistant *V. parahaemolyticus* isolates from retail (i.e., VHT77 and VHT78) and environment samples (i.e., VHT1 and VHT2) (8), and that the two-step enrichment technique of Meza did not successfully re-resuscitate VBNC-reverted *V. parahaemolyticus* isolates examined (VHT51, VHT52, VHT77, VHT78) (Table 8). Further, the Mizunoe's re-resuscitation method of using hydrogen peroxide degrading agents (2) did not contribute to the re-resuscitation of the lab strains (data not shown). This could be attributed to the differential intrinsic conditions (i.e., shellfish organisms) *V. parahaemolyticus* isolates were associated with prior to enrichment resuscitation procedures as described in various *Vibrio* species, including *V. parahaemolyticus* (43,44,45) and *V. parahaemolyticus* diversity (i.e., Mizunoe et al. vs. the lab strains in this study and Meza et al.) (2,8). The intrinsic conditions could possess growth factors in their symbiotic organisms which could be unavailable in the lab pure isolate culture conditions (i.e., during sub-culturing), thereby indicating the need for nutrient and non-defined growth factor availability by this bacterial group (i.e., resuscitated *V. parahaemolyticus* of regular and heat-resistant strains) to stay viable and culturable.

Microscopy of VBNC-reverted *V. parahaemolyticus* isolates, both the regular and heat-resistant strains, exhibited intact but morphologically distorted (i.e., oval/round) cells, hence, indicating cell viability (i.e., VBNC), cold storage distortion of cell morphology (46), and the Gram-staining limitation to distinguish the cellular morphology among the enriched, enriched + heat-selected, enriched + heat-selected + refrigeration, and BHI cultured cells of two groups of *V. parahaemolyticus* (i.e., the regular and heat-resistant strains) examined (Table 8).

Vibriosis is a persistent human health concern most likely attributed to the availability of VBNC *V. parahaemolyticus* sub-groups (i.e., heat-resistant, heat-dependent resuscitation strains) in contaminated seafood acquired by consumers following the safe seafood cooking procedures of USDA (14) and the compromising detection method of FDA (9). The two-step enrichment method of Meza et al. (8) discovered, for the first time, diverse heat-resistant *V. parahaemolyticus* in retail seafood, as demonstrated in their diverse growth phenotypes (i.e., in

this study, VHT77 vs VHT78; previous work, VHT1/VHT2 VS VHT77/VHT78) on sheep blood agar (Figure 4) (8); thereby, reconfirming the limitation of the pre-existing seafood examination and cooking protocols for seafood safety from *V. parahaemolyticus* contamination. Further investigation (in this study and Meza et al.) (8) suggests the availability of three thermally distinct *V. parahaemolyticus* groups (Table 2, Table 5) (i.e., regular, heat-resistant, and heat/pasteurization-resistant), that their heat-resistant phenotypes and prevalence could be environment-dependent (i.e., farm-raised vs wild-caught) (Table 2), that they could possess *tdh*-, *trh*-independent hemolysin, functional urease protein with manipulated gene sequence (i.e., rendered a gene-specific PCR amplification failure) (Figure 5, Figure 6, Table 6), non-detectable *tdh/trh* gene sequence manipulation by gene-specific PCR, that they (100% of tested strains) possess a MAR between 0.11-0.44 (Table 7) (11) that could pose a public health concern, especially seafood consumers infected by the pathogen, and that the two-step enrichment method of Meza et al. (8) and the hydrogen-peroxide induced re-resuscitation method of Mizunoe et al. (2) could not re-resuscitate the VBNC-reverted lab strains, including this study and Meza et al. (8) (Table 8).

5. CONCLUSION

Persistent vibriosis could be attributed to the underexplored biology of VBNC heat-resistant *V. parahaemolyticus* and consumption of seafood contaminated with *V. parahaemolyticus*, including this group, as a result of the repeated use of a failed investigation or cooking method. Both regular and heat-resistant *V. parahaemolyticus* coexist in farm-raised and wild-caught seafood, and the latter group (i.e., heat-resistant *V. parahaemolyticus*) could evade the existing detection (9) and cooking (14) protocol, thereby indicating the need for food safety investigation transformation from total *V. parahaemolyticus*. The present study validated the effectiveness of the modified two-step enrichment method of Meza to recover total *V. parahaemolyticus* from environment (8), wet-market (8), and retail (in this study) seafoods and suggest testing larger sizes and food varieties to warrant food safety from different forms of *V. parahaemolyticus* in seafoods and other emerging *V. parahaemolyticus*-associated foods, including dairy (47), and that adoption of this method by policymakers could improve detection accuracy and food safety from this pathogen. However, a novel method for re-resuscitating VBNC-reverted lab *V. parahaemolyticus* strains is anticipated to enable further investigation of viable and culturable *V. parahaemolyticus* lab strains, including the heat-resistant strains.

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