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Vibrio parahaemolyticus and *Vibrio vulnificus* Recovered from Oysters during an Oyster Relay Study

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ABSTRACT *Vibrio parahaemolyticus* and *Vibrio vulnificus* are naturally occurring estuarine bacteria and are the leading causes of seafood-associated infections and mortality in the United States. Though multiple-antibiotic-resistant *V. parahaemolyticus* and *V. vulnificus* strains have been reported, resistance patterns in vibrios are not as well documented as those of other foodborne bacterial pathogens. Salinity relaying (SR) is a postharvest processing (PHP) treatment to reduce the abundances of these pathogens in shellfish harvested during the warmer months. The purpose of this study was to evaluate the antimicrobial susceptibility (AMS), pathogenicity, and genetic profiles of *V. parahaemolyticus* and *V. vulnificus* recovered from oysters during an oyster relay study. Isolates (*V. parahaemolyticus* [$n = 296$] and *V. vulnificus* [$n = 94$]) were recovered from oysters before and during the 21-day relaying study to detect virulence genes (*tdh* and *trh*) and genes correlated with virulence (*vcgC*) using multiplex quantitative PCR (qPCR). AMS to 20 different antibiotics was investigated using microbroth dilution, and pulsed-field gel electrophoresis (PFGE) was used to study the genetic profiles of the isolates. Twenty percent of *V. vulnificus* isolates were *vcgC*$^+$, while 1 and 2% of *V. parahaemolyticus* were *tdh*$^+$ and *trh*$^+$, respectively. More than 77% of the *V. vulnificus* isolates and 30% of the *V. parahaemolyticus* isolates were resistant to at least one antimicrobial. Forty-eight percent of *V. vulnificus* and 8% of *V. parahaemolyticus* isolates were resistant to two or more antimicrobials. All isolates demonstrated a high genetic diversity, even among those isolated from the same site and having a similar AMS profile. No significant effects of the relaying process on AMS, virulence genes, or PFGE profiles of *V. vulnificus* and *V. parahaemolyticus* were observed.

IMPORTANCE Analysis of the antibiotic resistance profiles of *V. vulnificus* and *V. parahaemolyticus* isolated from oysters during this study indicated that more than 48% of *V. vulnificus* isolates were resistant to two or more antimicrobials, including those recommended by the CDC for treating *Vibrio* infections. Also, the *V. parahaemolyticus* isolates showed high MICs for some of the *Vibrio* infection treatment antibiotics. Monitoring of AMS profiles of this bacterium is important to ensure optimal treatment of infections and improve food safety. Our study showed no significant differences in the AMS profiles of *V. vulnificus* ($P = 0.26$) and *V. parahaemolyticus* ($P = 0.23$) isolated from the oysters collected before versus after relaying. This suggests that the salinity of the relaying sites did not affect the AMS profiles of the *Vibrio* isolates, although it did reduce the numbers of these bacteria in oysters (S. Parveen et al., J Food Sci 82:484–491, 2017, https://doi.org/10.1111/1750-3841.13584).

KEYWORDS oysters, relay, *Vibrio parahaemolyticus*, *Vibrio vulnificus*

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Vibrio parahaemolyticus and *Vibrio vulnificus*, naturally occurring estuarine bacteria, are the leading causes of seafood-borne mortality and illness in the United States (1–3). *V. parahaemolyticus* causes the highest number of seafood-associated bacterial gastroenteritis in the United States and in Asian countries (4–6). The primary classification of *V. parahaemolyticus* is a serotyping scheme, which depends mainly on the antigenic properties of the somatic (O) and capsular (K) antigens (7). *V. parahaemolyticus* is a multiserogroup pathogen, with at least 13 O serogroups and 71 K serotypes detected (8, 9).

Thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) are two major virulence factors associated with *V. parahaemolyticus* which are closely related to its pathogenicity (10). Epidemiological investigations indicated that *tdh* is one of the major pathogenic factors in *V. parahaemolyticus* and is prevalent in almost all (95%) clinical isolates (11, 12).

Vibrio vulnificus is responsible for more than 95% of seafood-related deaths in the United States (13), especially among high-risk consumers with immunocompromised conditions or liver disease (14, 15). Despite the high number of at-risk consumers and the fact that in some regions, oysters during the summer months typically contain 10^3 to 10^4 CFU/g of *V. vulnificus*, the incidence of disease is relatively low (16–19), leading to the hypothesis that not all strains of *V. vulnificus* are equally virulent. Several biomarkers, e.g., the virulence-correlated gene (*vcg*), 16S rRNA, and the capsular polysaccharide operon (CPS), have been used to differentiate virulent- from nonvirulent-type *V. vulnificus* strains (20). There are, however, no available molecular markers with sufficient resolving power to categorize with absolute certainty the pathogenicity of *V. vulnificus* strains (17). Rosche et al. (21) reported that 90% of the C-type strains were clinical isolates (*vcgC* positive), while 93% of environmental isolates were classified as E-type (*vcgC* negative). Therefore, *vcgC* may serve as a reliable biomarker to screen for potentially virulent *V. vulnificus* strains.

According to the U.S. Centers for Disease Control and Prevention (CDC), the incidence of *Vibrio* infections has increased since 2001 (almost tripling during the period between 1996 and 2010), while all other foodborne infection rates have remained the same or decreased (13). Also, in 2013, the FoodNet found that the incidence of *Vibrio* infections was significantly (32%) higher than that during 2010 to 2012 (22). In the severe form of *V. vulnificus* infection (septicemia), the fatality rates for patients significantly increased with the delays between the onset of illness and initiation of antibiotic treatment (14); the awareness of antimicrobial resistance of these two pathogens is not as well documented as those for other foodborne bacterial pathogens. *Vibrio* spp. are susceptible to most antimicrobial agents of veterinary and human significance (3). Recent studies indicated that *V. parahaemolyticus* and *V. vulnificus* have developed multiple antimicrobial resistances, which may be due to the discharging of wastewater containing pathogenic bacteria with antimicrobial resistance genes (23). Among individuals consuming raw or undercooked contaminated seafood and seafood products, this can lead to serious public health issues (24).

Because of health concerns associated with the consumption of raw product, the National Shellfish Sanitation Program (NSSP) provides dealers the option to use an approved and validated postharvest processing (PHP) method to reduce *Vibrio* levels and make safety-added labeling claims. The few studies performed on the efficacy of relaying and depuration as PHP methods for reducing the abundance of *Vibrio* bacteria, especially *V. vulnificus*, in oysters have shown promising results. Interestingly, flow-through depuration was successful in reducing *V. vulnificus* in artificially contaminated oysters from $>10^4$ most probable number (MPN)/g to <30 MPN/g when incoming water salinity was higher than (30 ppt) after 6 days (25). Recently, we reported that relaying of oysters to high-salinity field sites (29 to 34 ppt) or transfer to high-salinity recirculating aquaculture systems (RAS) (32 to 34 ppt) can reduce average levels of these bacteria in oysters by 2 to 5 logs after 21 to 28 days. These methods were more effective in reducing *V. vulnificus* than *V. parahaemolyticus*. Oyster mortality rates averaged 4% or less and did not exceed 7% (26).

TABLE 1 *vcgC*-positive *Vibrio vulnificus* isolated during the high- and moderate-salinity relaying process

Day/site[a]	No. of isolates	% *vcgC*$^+$
0	36	8.3
7/S1	36	16.6
14/S1	37	27.0
21/S1	27	40.7
7/S2	47	17.0
14/S2	43	18.6
21/S2	37	24.3

[a]S1, moderate-salinity site, 12 to 18.7 ppt; S2, high-salinity site, 28 to 32.5 ppt.

It has been reported that salinity can affect the growth and survival of *V. parahaemolyticus* in aquatic environments (26, 27**)**. Whitaker et al. (28) investigated the growth of *V. parahaemolyticus* at different salt concentrations and observed that salt can affect its response to pH and temperature. The same study also reported that the cytotoxic effects of *V. parahaemolyticus* in human intestinal cells were greater when this bacterium was grown in a medium containing 1% salt than with 3% salt. However, it is undetermined whether or not high-salinity relaying has any appreciable effect on the prevalence of pathogenic strains (virulence genes), antibiotic resistance, and genetic profiles of *V. parahaemolyticus* and *V. vulnificus*.

In this study, we evaluated the phenotypic and genotypic characteristics of PCR-confirmed *Vibrio* isolates recovered from oyster samples before and during the 21-day relaying process. The objectives of this study were to (i) investigate the antimicrobial susceptibility profiles of the PCR-confirmed *V. parahaemolyticus* and *V. vulnificus* isolates, as well as the predominant O serogroups of *V. parahaemolyticus* recovered during the relaying process; (ii) study the virulence properties of the *V. parahaemolyticus* and *V. vulnificus* strains isolated during the relaying trials; and (iii) investigate the genetic relationships of these isolates based on their susceptibility profiles using the pulsed-field gel electrophoresis (PFGE) technique.

RESULTS

Virulence genes of *V. vulnificus* and *V. parahaemolyticus* isolates. In this study, the *vcgC* gene of *V. vulnificus* isolated from oyster samples was detected. Fifty-five of 263 (20.9%) PCR-confirmed *V. vulnificus* isolates were *vcgC*$^+$; specifically, 27 of 100 (27%) tested isolates from the moderate-salinity site and 25 of 127 (19.7%) tested isolates from the high-salinity site were *vcgC*$^+$ (Table 1). There was no significant difference between the frequencies of detection of the virulence gene between isolates from the moderate- and the high-salinity sites.

The *V. parahaemolyticus* multiplex PCR assay was used to detect the presence of *tdh*$^+$ and/or *trh*$^+$ genes on PCR-confirmed *V. parahaemolyticus* isolated from oyster samples during the relaying trials. Out of 278 tested isolates, only two of them were *tdh*$^+$ (0.72%), and six isolates were *trh*$^+$ (2.2%) (Table 2). Similar to *V. vulnificus*, we observed no difference between the frequencies of detection of the pathogenicity markers of *V. parahaemolyticus* isolates at relaying sites of differing salinity.

TABLE 2 *tdh*- and *trh*-positive *V. parahaemolyticus* isolated during the high- and moderate-salinity relaying process

Day/site[a]	No. of isolates	% *tdh*$^+$	% *trh*$^+$
0	37	0	8.1
7/S1	34	0	5.9
14/S1	35	2.9	0
21/S1	36	0	0
7/S2	47	0	2.1
14/S2	43	0	0
21/S2	46	2.2	0

[a]S1, moderate-salinity site, 12 to 18.7 ppt; S2, high-salinity site, 28 to 32.5 ppt.

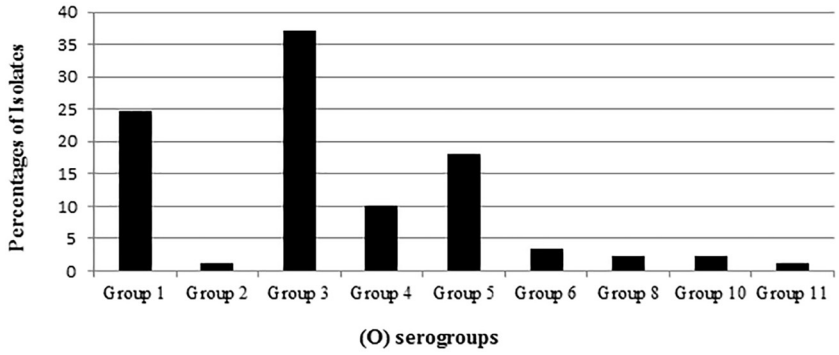


FIG 1 Somatic (O) serogroups of *Vibrio parahaemolyticus* isolated during an oyster relay study.

Identification of the O serogroups of *V. parahaemolyticus* isolates. Among the 92 *V. parahaemolyticus* isolates obtained from the oysters during the relaying study, 3 were untypeable. The most predominant O serogroups among the 89 typeable *V. parahaemolyticus* isolates were 3 and 1, as they represent 37% and 25% of the isolates, respectively, while none of the isolates belonged to serogroups 7, 9, 12, and 13 (Fig. 1).

Prevalence of antimicrobial resistance in *V. vulnificus*. All tested *V. vulnificus* isolates were uniformly susceptible to four of the 20 tested antibiotics, two of which were recommended by the CDC for treating *Vibrio* infections (ciprofloxacin, levofloxacin, piperacillin, and piperacillin-tazobactam) (Table 3). More than 77% of *V. vulnificus* isolates were resistant to at least one antimicrobial, and more than 48% were resistant to two or more antimicrobials (see Appendix SA-1 in the supplemental material). Ten percent of tested isolates were found to be completely susceptible to all antimicrobials tested.

Overall, the most common resistances were those to cephalothin (67%), tetracycline (29%), amoxicillin-clavulanic acid, and ampicillin (26%). With regard to the recommended antibiotics for the treatment of *V. vulnificus* infections, *V. vulnificus* tested isolates exhibited resistance against ceftriaxone (12%), ceftazidime (12%), and cefotaxime (7%), a third-generation cephalosporin. Within the tetracycline class of antibi-

TABLE 3 Antimicrobial resistance patterns among *V. vulnificus* strains isolated during the oyster relaying process

	% with profile[a]:		
Antibiotic	**S**	**I**	**R**
Amikacin[b]	88	12	0
Amoxicillin-clavulanic acid	66	9	26
Ampicillin	72	2	26
Ampicillin-sulbactam	93	3	3
Cefepime	93	5	2
Cefotaxime[b]	91	2	7
Cefoxitin	36	47	17
Ceftazidime[b]	72	16	12
Ceftriaxone[b]	78	10	12
Cephalothin	31	2	67
Chloramphenicol	86	12	2
Ciprofloxacin[b]	100	0	0
Doxycycline[b]	71	9	21
Imipenem	76	9	16
Levofloxacin[b]	100	0	0
Meropenem	95	5	0
Piperacillin	100	0	0
Piperacillin-tazobactam	100	0	0
Tetracycline[b]	64	7	29
Trimethoprim-sulfamethoxazole[b]	98	0	2

[a]S, susceptible; I, intermediate; R, resistant.
[b]CDC-recommended antibiotic for *Vibrio* infections.

TABLE 4 Antimicrobial resistance patterns among *Vibrio parahaemolyticus* strains isolated during the oyster relaying process

Antibiotic	% with profile[a]:		
	S	I	R
Amikacin[b]	96	3	1
Amoxicillin-clavulanic acid	99	0	1
Ampicillin	91	5	3
Ampicillin-sulbactam	100	0	0
Cefepime	95	4	1
Cefotaxime[b]	97	0	3
Cefoxitin	92	25	13
Ceftazidime[b]	87	4	7
Ceftriaxone[b]	90	4	5
Cephalothin	41	48	11
Chloramphenicol	99	1	0
Ciprofloxacin[b]	100	0	0
Doxycycline[b]	98	1	1
Imipenem	97	3	0
Levofloxacin[b]	100	0	0
Meropenem	98	2	0
Piperacillin	98	1	1
Piperacillin-tazobactam	100	0	0
Tetracycline[b]	99	0	1
Trimethoprim-sulfamethoxazole[b]	100	0	0

[a]S, susceptible; I, intermediate; R, resistant.
[b]CDC-recommended antibiotic for *Vibrio* infections.

otics, isolates exhibited high resistance to tetracycline (29%) and doxycycline (21%). Also, 2% resistance was observed against trimethoprim-sulfamethoxazole. Intermediate resistance was expressed against ceftazidime (16%), amikacin (12%), ceftriaxone (10%), doxycycline, and tetracycline (7%). Quinolones (ciprofloxacin and levofloxacin) were the only recommended class of drug to which all *V. vulnificus* isolates were completely susceptible. *V. vulnificus* displayed the highest percentage of intermediate resistance (47%) to cefoxitin (Table 3).

Of the 11 isolates positive for *vcgC*, 8 isolates displayed resistance to one or more of the tested antibiotics, and 7 isolates expressed resistance to more than 4 tested antibiotics (Appendix SA-1).

Prevalence of antimicrobial resistance in *V. parahaemolyticus*. All tested isolates were susceptible to five of the 20 tested antibiotics, including four of those recommended by the CDC for treating *Vibrio* infections (ciprofloxacin, levofloxacin, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole) (Table 4). Only about 8% of the isolates showed resistance to two or more tested antibiotics (Appendix SA-2).

Overall, all *V. parahaemolyticus* isolates were susceptible or expressed low resistance and intermediate resistance to CDC-recommended antibiotics for *Vibrio* treatment; the exception was ceftazidime, as 9% of the isolates displayed resistance to this antibiotic. However, even the low percentage of resistance displayed by *V. parahaemolyticus* isolates to other cephalosporin antibiotics (3% for cefotaxime and 5% for ceftriaxone) might be cause for concern, as the cephalosporins are considered to be one of the best defenses against severe *Vibrio* infections (29). The highest resistance (13%) in *V. parahaemolyticus* isolates was for cefoxitin, followed by cephalothin (11%), ceftazidime (5%), and cefotaxime (3%), while 48% of the isolates were characterized by intermediate resistance to cephalothin, followed by cefoxitin (25%) (Table 4).

Among the *tdh*$^+$ and/or *trh*$^+$ *V. parahaemolyticus* isolates, the *tdh*$^+$ isolates expressed no resistance to any tested antibiotics and intermediate resistance to cephalothin. Each of the *trh*$^+$ isolates was resistant to one of the tested antibiotics (ampicillin and piperacillin) and expressed intermediate resistance to more than one antibiotic (Appendix SA-2). In general, none of the four pathogenic isolates showed multiple resistance, and all of them showed intermediate resistance to cephalothin.

Comparison between *V. vulnificus* and *V. parahaemolyticus* antimicrobial resistance and intermediate resistance. *V. vulnificus* isolates in this study possessed a

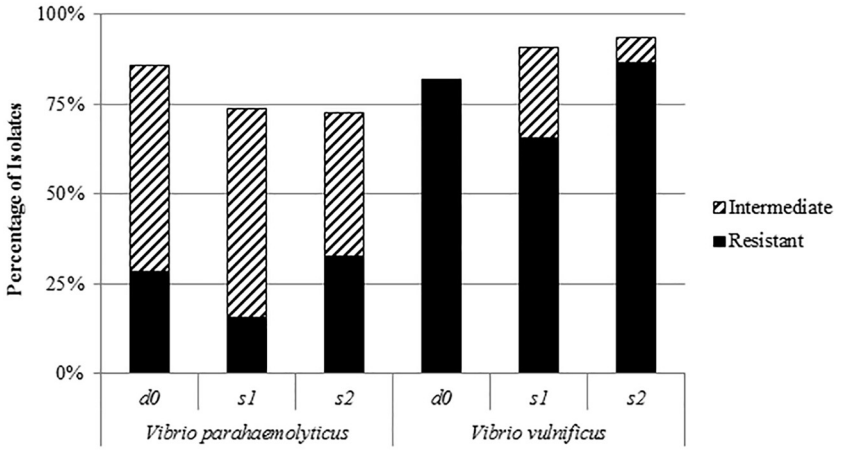


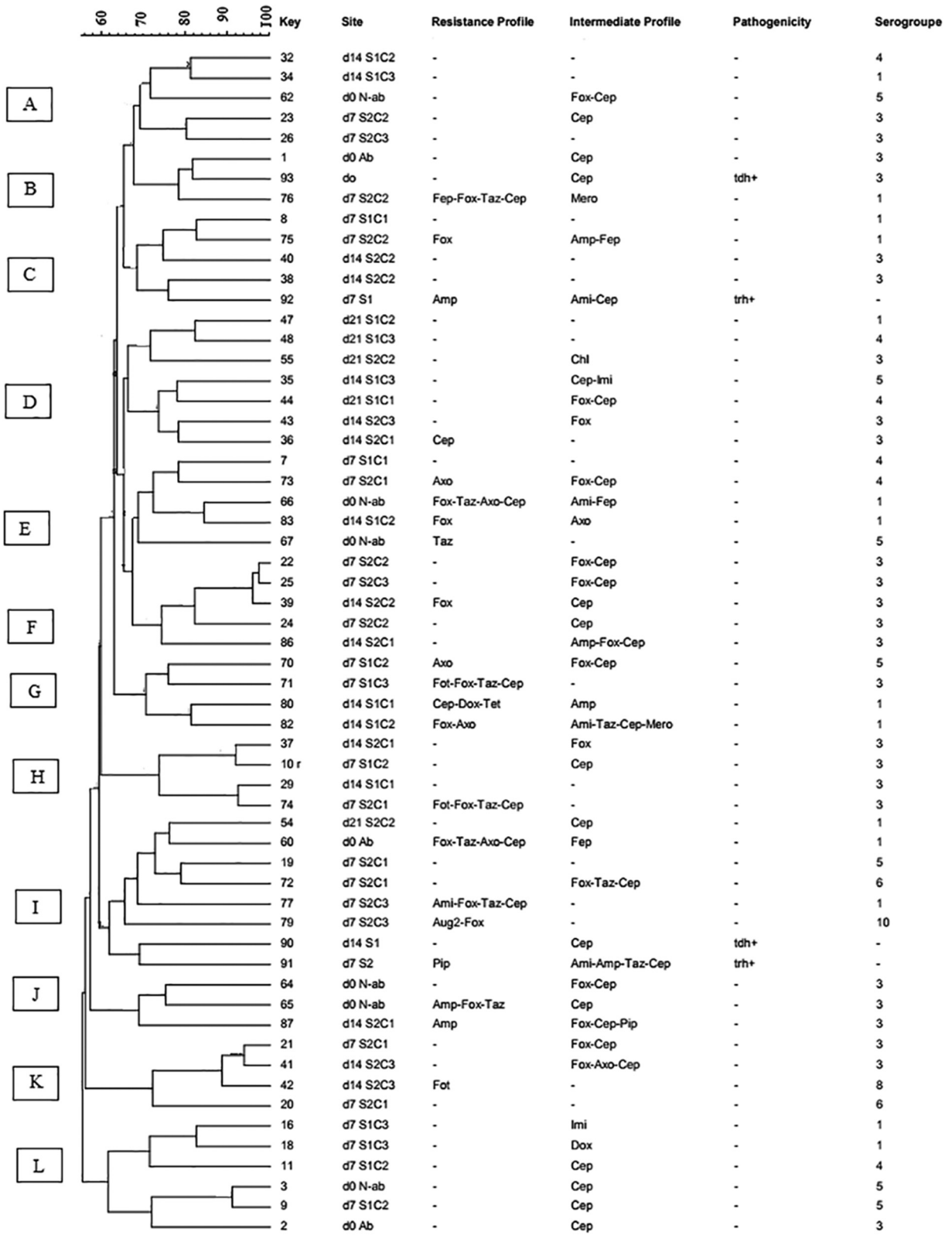
FIG 2 Percentages of antibiotic resistance and intermediate resistance of *Vibrio vulnificus* and *Vibrio parahaemolyticus* isolated from different sites. *d0*, harvesting day; *s1*, moderate-salinity site; *s2*, high-salinity site.

higher resistance to all tested antibiotics, including those recommended by the CDC for *Vibrio* infections (Fig. 2), than did *V. parahaemolyticus* isolates (Fig. 3). Also, *V. vulnificus* isolates expressed higher intermediate resistance to all tested antibiotics except cephalothin (Fig. 2). More than 48% of *V. vulnificus* isolates were resistant to two or more antimicrobials, while only 18% of them were susceptible to all tested antibiotics. On the other hand, only about 8% of *V. parahaemolyticus* isolates showed resistance to two or more tested antibiotics, and approximately 54% of them were susceptible to all tested antibiotics.

The results of this study showed that there were no significance differences between the antimicrobial susceptibility profiles of *V. vulnificus* ($P = 0.26$) and *V. parahaemolyticus* ($P = 0.23$) isolated from the oysters before the relaying process (day 0) and after relaying to the two relaying sites, site 1 (moderate salinity) and site 2 (high salinity) (Fig. 4).

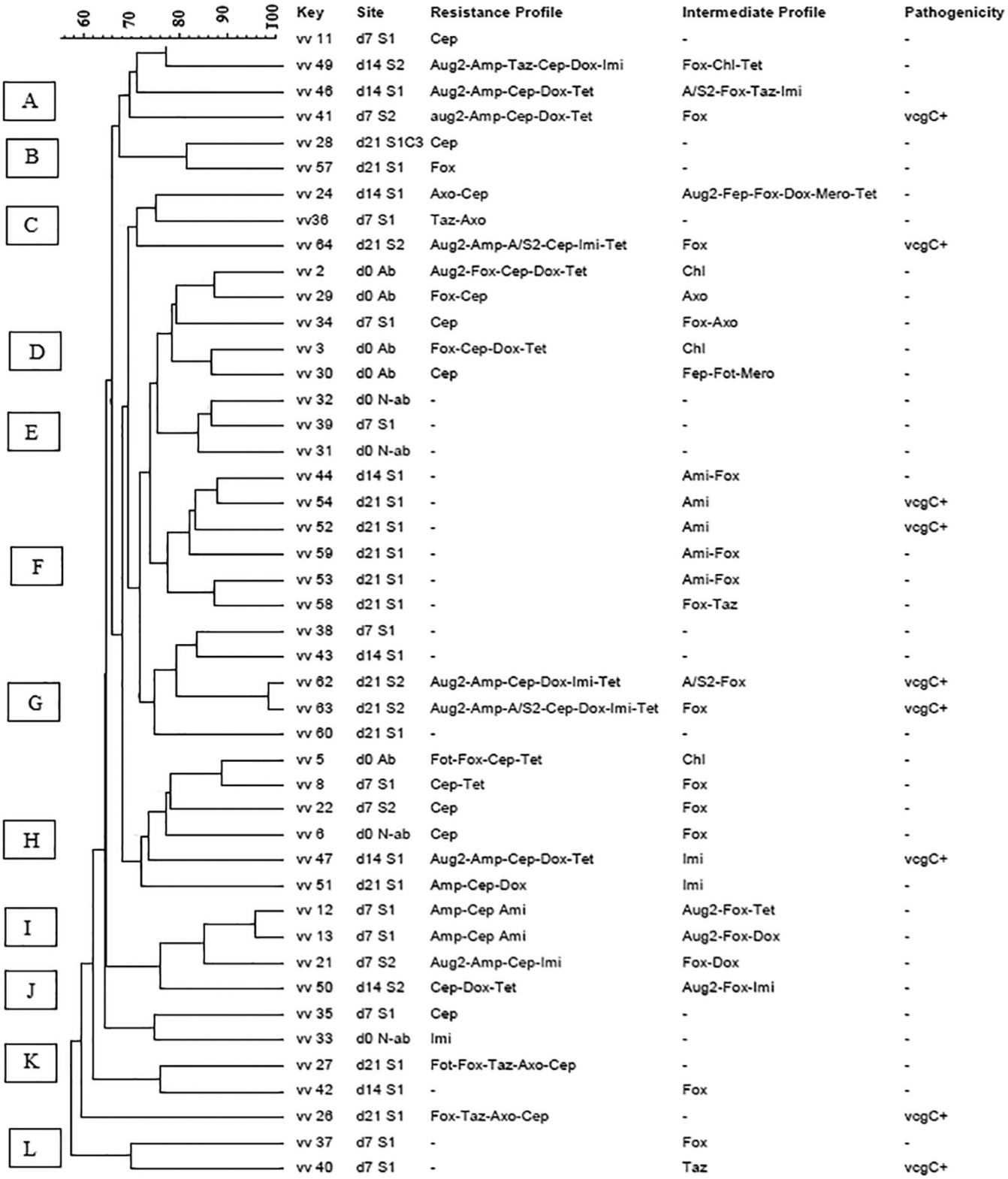
Characterization of *V. parahaemolyticus* and *V. vulnificus* isolated from oysters using pulsed-field gel electrophoresis. (i) PFGE analysis of *V. parahaemolyticus* isolates. In this study, PFGE was performed using a restriction enzyme (SfiI) to study the genetic relatedness between *V. parahaemolyticus* isolates, selected based on their antimicrobial susceptibility profiles and location of isolation during the relaying trials from the harvesting and the two relaying sites (moderate- and high-salinity sites). A total of 58 PFGE banding patterns were generated from 64 *V. parahaemolyticus* strains isolated from high- and moderate-salinity relaying trials. The dendrogram revealed 12 clusters (A to L) with 60% pattern similarity (Fig. 3), indicating extensive genetic diversity among the isolates, even between those obtained from the same site, same relaying interval day, and with similar antibiotic susceptibility profiles. However, a few tested isolates showed a tendency to cluster based on their antimicrobial susceptibility profiles and the site of isolation. For example, clusters A (5 isolates), D (7 isolates), K (6 isolates), and F (5 isolates) showed mostly no resistance to any of the tested antibiotics and intermediate resistance to cephalothin. Clusters E (5 isolates) and G (4 isolates) showed resistance or intermediate resistance to one or more tested antibiotics. Overall, the PFGE profiles of *V. parahaemolyticus* were genetically diverse and had no relationship according to the sampling site (relaying process), antimicrobial susceptibility test results, or pathogenicity.

(ii) PFGE analysis of *V. vulnificus* isolates. A dendrogram was constructed based on SfiI PFGE patterns (Fig. 4) to compare the banding profiles of *V. vulnificus* isolates with different antimicrobial susceptibility and pathogenicity profiles isolated from oysters during the moderate- and high-salinity relaying process. Out of the 58 isolates of *V. vulnificus* examined in this study, 47 isolates were successfully characterized by the



	Key	Site	Resistance Profile	Intermediate Profile	Pathogenicity	Serogroupe
A	32	d14 S1C2	-	-	-	4
	34	d14 S1C3	-	-	-	1
	62	d0 N-ab	-	Fox-Cep	-	5
	23	d7 S2C2	-	Cep	-	3
	26	d7 S2C3	-	-	-	3
B	1	d0 Ab	-	Cep	-	3
	93	do	-	Cep	tdh+	3
	76	d7 S2C2	Fep-Fox-Taz-Cep	Mero	-	1
C	8	d7 S1C1	-	-	-	1
	75	d7 S2C2	Fox	Amp-Fep	-	1
	40	d14 S2C2	-	-	-	3
	38	d14 S2C2	-	-	-	3
	92	d7 S1	Amp	Ami-Cep	trh+	-
D	47	d21 S1C2	-	-	-	1
	48	d21 S1C3	-	-	-	4
	55	d21 S2C2	-	Chl	-	3
	35	d14 S1C3	-	Cep-Imi	-	5
	44	d21 S1C1	-	Fox-Cep	-	4
	43	d14 S2C3	-	Fox	-	3
	36	d14 S2C1	Cep	-	-	3
E	7	d7 S1C1	-	-	-	4
	73	d7 S2C1	Axo	Fox-Cep	-	4
	66	d0 N-ab	Fox-Taz-Axo-Cep	Ami-Fep	-	1
	83	d14 S1C2	Fox	Axo	-	1
	67	d0 N-ab	Taz	-	-	5
F	22	d7 S2C2	-	Fox-Cep	-	3
	25	d7 S2C3	-	Fox-Cep	-	3
	39	d14 S2C2	Fox	Cep	-	3
	24	d7 S2C2	-	Cep	-	3
	86	d14 S2C1	-	Amp-Fox-Cep	-	3
G	70	d7 S1C2	Axo	Fox-Cep	-	5
	71	d7 S1C3	Fot-Fox-Taz-Cep	-	-	3
	80	d14 S1C1	Cep-Dox-Tet	Amp	-	1
	82	d14 S1C2	Fox-Axo	Ami-Taz-Cep-Mero	-	1
H	37	d14 S2C1	-	Fox	-	3
	10 r	d7 S1C2	-	Cep	-	3
	29	d14 S1C1	-	-	-	3
	74	d7 S2C1	Fot-Fox-Taz-Cep	-	-	3
I	54	d21 S2C2	-	Cep	-	1
	60	d0 Ab	Fox-Taz-Axo-Cep	Fep	-	1
	19	d7 S2C1	-	-	-	5
	72	d7 S2C1	-	Fox-Taz-Cep	-	6
	77	d7 S2C3	Ami-Fox-Taz-Cep	-	-	1
	79	d7 S2C3	Aug2-Fox	-	-	10
	90	d14 S1	-	Cep	tdh+	-
	91	d7 S2	Pip	Ami-Amp-Taz-Cep	trh+	-
J	64	d0 N-ab	-	Fox-Cep	-	3
	65	d0 N-ab	Amp-Fox-Taz	Cep	-	3
	87	d14 S2C1	Amp	Fox-Cep-Pip	-	3
K	21	d7 S2C1	-	Fox-Cep	-	3
	41	d14 S2C3	-	Fox-Axo-Cep	-	3
	42	d14 S2C3	Fot	-	-	8
	20	d7 S2C1	-	-	-	6
L	16	d7 S1C3	-	Imi	-	1
	18	d7 S1C3	-	Dox	-	1
	11	d7 S1C2	-	Cep	-	4
	3	d0 N-ab	-	Cep	-	5
	9	d7 S1C2	-	Cep	-	5
	2	d0 Ab	-	Cep	-	3

FIG 3 Dendrogram of PFGE profiles of *Vibrio parahaemolyticus* isolates. d0, d7, d14, and d21, harvesting days 0, 7, 14, and 21, respectively; S1, moderate-salinity site; S2, high-salinity site; C, oyster composite; *tdh*, thermostable direct hemolysin; *trh*, TDH-related hemolysis; Ami, amikacin; Aug2, amoxicillin-clavulanic acid; Amp, ampicillin; Fep, cefepime; Fot, cefotaxime; Fox, cefoxitin; Taz, ceftazidime; Axo, ceftriaxone; Cep, cephalothin; Chl, chloramphenicol; Mero, meropenem; Pip, piperacillin. Minus signs in the resistance profile column indicate susceptibility to all tested antibiotics or untypeable, and those in the pathogenicity column indicate negativity for *tdh* or *trh*. Letters A to L on the left represent pulsed-field gel electrophoresis clusters; −. untypeable; Ab, temperature-abused oyster; N-ab, non-abused oyster.



Key	Site	Resistance Profile	Intermediate Profile	Pathogenicity
vv 11	d7 S1	Cep	-	-
vv 49	d14 S2	Aug2-Amp-Taz-Cep-Dox-Imi	Fox-Chl-Tet	-
vv 46	d14 S1	Aug2-Amp-Cep-Dox-Tet	A/S2-Fox-Taz-Imi	-
vv 41	d7 S2	aug2-Amp-Cep-Dox-Tet	Fox	vcgC+
vv 28	d21 S1C3	Cep	-	-
vv 57	d21 S1	Fox	-	-
vv 24	d14 S1	Axo-Cep	Aug2-Fep-Fox-Dox-Mero-Tet	-
vv36	d7 S1	Taz-Axo	-	-
vv 64	d21 S2	Aug2-Amp-A/S2-Cep-Imi-Tet	Fox	vcgC+
vv 2	d0 Ab	Aug2-Fox-Cep-Dox-Tet	Chl	-
vv 29	d0 Ab	Fox-Cep	Axo	-
vv 34	d7 S1	Cep	Fox-Axo	-
vv 3	d0 Ab	Fox-Cep-Dox-Tet	Chl	-
vv 30	d0 Ab	Cep	Fep-Fot-Mero	-
vv 32	d0 N-ab	-	-	-
vv 39	d7 S1	-	-	-
vv 31	d0 N-ab	-	-	-
vv 44	d14 S1	-	Ami-Fox	-
vv 54	d21 S1	-	Ami	vcgC+
vv 52	d21 S1	-	Ami	vcgC+
vv 59	d21 S1	-	Ami-Fox	-
vv 53	d21 S1	-	Ami-Fox	-
vv 58	d21 S1	-	Fox-Taz	-
vv 38	d7 S1	-	-	-
vv 43	d14 S1	-	-	-
vv 62	d21 S2	Aug2-Amp-Cep-Dox-Imi-Tet	A/S2-Fox	vcgC+
vv 63	d21 S2	Aug2-Amp-A/S2-Cep-Dox-Imi-Tet	Fox	vcgC+
vv 60	d21 S1	-	-	-
vv 5	d0 Ab	Fot-Fox-Cep-Tet	Chl	-
vv 8	d7 S1	Cep-Tet	Fox	-
vv 22	d7 S2	Cep	Fox	-
vv 6	d0 N-ab	Cep	Fox	-
vv 47	d14 S1	Aug2-Amp-Cep-Dox-Tet	Imi	vcgC+
vv 51	d21 S1	Amp-Cep-Dox	Imi	-
vv 12	d7 S1	Amp-Cep Ami	Aug2-Fox-Tet	-
vv 13	d7 S1	Amp-Cep Ami	Aug2-Fox-Dox	-
vv 21	d7 S2	Aug2-Amp-Cep-Imi	Fox-Dox	-
vv 50	d14 S2	Cep-Dox-Tet	Aug2-Fox-Imi	-
vv 35	d7 S1	Cep	-	-
vv 33	d0 N-ab	Imi	-	-
vv 27	d21 S1	Fot-Fox-Taz-Axo-Cep	-	-
vv 42	d14 S1	-	Fox	-
vv 26	d21 S1	Fox-Taz-Axo-Cep	-	vcgC+
vv 37	d7 S1	-	Fox	-
vv 40	d7 S1	-	Taz	vcgC+

FIG 4 Dendrogram of PFGE profiles of *Vibrio vulnificus* (vv) isolates. d0, d7, d14, and d21, harvesting days 0, 7, 14, and 21, respectively; S1, moderate-salinity site; S2, high-salinity site; C, oyster composite; *vcgC*, virulence-correlating gene type C; Ami, amikacin; Aug2, amoxicillin-clavulanic acid; Amp, ampicillin; A/S2, ampicillin-sulbactam; Fep, cefepime; Fot, cefotaxime; Fox, cefoxitin; Taz, ceftazidime; Axo, ceftriaxone; Cep, cephalothin; Chl, chloramphenicol; Dox, doxycycline; Imi, imipenem; Mero, meropenem; Pip, piperacillin; Tet, tetracycline. Minus signs in the resistance profile column indicate susceptibility to all tested antibiotics, and those in the pathogenicity column indicate negativity for *vcgC*. Letters A to K on the left represent pulsed-field gel electrophoresis clusters. Ab, temperature-abused oyster; N-ab, non-abused oyster.

PFGE, and approximately 19% of the isolates were not typeable and failed to yield discernible PFGE patterns. A total of 45 PFGE banding patterns were generated from 47 *V. vulnificus* isolates. These generated patterns were grouped into 11 clusters (A to K) with about 60% similarity (Fig. 4). *V. vulnificus* strains isolated from different sites with different antimicrobial susceptibility and pathogenicity profiles were distributed among all identified clusters. Similar to *V. parahaemolyticus*, the PFGE profiles of *V. vulnificus* were genetically diverse, and no genetic relationship was observed between the PFGE profile of this pathogen and the salinity relaying process.

DISCUSSION

In this study, we examined the presence of the *vcgC* gene in *V. vulnificus* as well as the presence of *tdh* and/or *trh* genes in *V. parahaemolyticus* isolated from oysters. Among all tested *V. vulnificus* isolates, 20.9% were *vcgC*$^+$, and no significant differences in the prevalences of the virulence genes were observed at the relaying sites of differing salinity. Our results were comparable to the findings of Warner and Oliver (19), who found that 15.6% of the *V. vulnificus* isolates recovered from oyster samples from the eastern coast of North Carolina were *vcgC*$^+$. We observed that only 0.72% of the *V. parahaemolyticus* isolates were *tdh*$^+$, and only 2.2% were *trh*$^+$. This is consistent with findings of DePaola et al. (30), who reported that *tdh*$^+$ and/or *trh*$^+$ isolates typically represent $<$1% of environmental *V. parahaemolyticus* strains, except in the Pacific Northwest. The prevalence of these strains may vary by sample type and detection method, as well as by location (31–33). Whitaker et al. (28) studied the effect of salinity on *V. parahaemolyticus* pathogenicity using the cytotoxicity assay, and they found that *V. parahaemolyticus* grown in 1% NaCl was significantly more cytotoxic than that grown in 3% NaCl. However, in this study, no clear differences in the prevalences of *V. parahaemolyticus* virulence genes at relaying sites of different salinity were observed; also, similar to our study, Johnson et al. (34) found that salinity was not a significant predictor of *tdh* density in oyster samples, but it was a significant predictor of the *trh* density.

In this study, *V. parahaemolyticus* isolates belonging to different O serogroups were frequently found to be isolated from the same site (Fig. 3), indicating the high degree of divergence demonstrated by this pathogen (7, 35). Most of the *V. parahaemolyticus* isolates in this study (37%) were found to belong to the O3 serogroup. Consequently, given the increase of gastroenteritis outbreaks associated with serotype O3:K6 globally (36–39), the capsular (K) antigens of these isolates should be tested to clearly investigate the abundance of this serotype among the *V. parahaemolyticus* strains isolated from Chesapeake Bay region.

Most of the *V. vulnificus* isolates recovered during the relaying process were resistant to at least one antimicrobial, and almost 50% of them were resistant to two or more antimicrobials. Ten percent of the tested isolates were found to be completely susceptible to all antimicrobials tested. This result is comparable with that of a recent study conducted to investigate the antimicrobial susceptibility of *V. vulnificus* isolates recovered from recreational and commercial water of Chesapeake Bay. They reported that only 12.5% of the *V. vulnificus* isolates were susceptible to all tested antimicrobials (40). Similar to our findings, 45% of the environmental *V. vulnificus* isolates from a study in South Carolina and Georgia estuaries were resistant to three or more classes of antibiotic agents, including those that are usually prescribed for *V. vulnificus* infections (41).

The CDC-recommended antibiotics for treating *V. vulnificus* infections are doxycycline, cephalosporins (e.g., ceftazidime), fluoroquinolones (such as levofloxacin, ciprofloxacin, or gatifloxacin), and trimethoprim-sulfamethoxazole plus an aminoglycoside (29). Tetracycline or ciprofloxacin can be used only in cases of severe or prolonged *Vibrio* infections (42). In this study, *V. vulnificus* tested isolates exhibited varied degrees of resistance and intermediate resistance against most of these antimicrobial agents, while the quinolones (ciprofloxacin and levofloxacin) were the only recommended class of drug to which all *V. vulnificus* isolates were completely susceptible. Compatible with our findings, Wong et al. (43) recently studied the *in vivo* efficacy of antibiotics for lethal *Vibrio* species, and they found that treatment of *V. vulnificus* infection that includes a

quinolone is associated with lower mortality. Shaw et al. (40) also reported that all tested *V. vulnificus* isolates were susceptible to quinolones. However, in contrast to our findings, all of their tested isolates were susceptible to tetracycline, and only 2% and 1% of their tested isolates exhibited intermediate resistance against ceftazidime and amikacin, respectively.

A large-scale study conducted to investigate the antimicrobial susceptibilities of both *V. parahaemolyticus* and *V. vulnificus* in Louisiana Gulf and retail oysters showed that *V. vulnificus* isolates were susceptible to the majority of tested antibiotics (20). However, the results of their study agreed with the present study and confirmed that fluoroquinolones were highly effective against *V. vulnificus* infection.

In our study, all *V. parahaemolyticus* isolates were susceptible or expressed low or intermediate resistance to the antibiotics (except for ceftazidime) recommended by the CDC for *Vibrio* infection treatment. In addition, approximately 8% of *V. parahaemolyticus* isolates showed resistances to two or more tested antibiotics, and approximately 54% were susceptible to all tested antibiotics. Shaw et al. (40) and Han et al. (20) reported for Chesapeake Bay and Gulf Coast oysters, respectively, a higher level of resistance among *V. parahaemolyticus* than among *V. vulnificus* isolates.

Our study showed no significant differences in the antimicrobial susceptibility profiles of *V. vulnificus* and *V. parahaemolyticus* isolated from the oysters before and during the relaying in both sites. This suggests that the salinity of the relaying sites did not impact the antimicrobial susceptibility profiles of the *Vibrio* isolates, although it reduced the overall concentration of these bacteria in oysters (26).

Molecular typing of *V. parahaemolyticus* has been shown to be a useful tool for tracking the source of infection and detection of virulent strains, as well as for determining the geographical and host distribution of possible variants (44). Studies focusing on the interspecies variability and genetic relationships among the environmental isolates are rare and limited to specific geographic areas (45). In this study, PFGE was conducted to determine the genetic relatedness between *V. parahaemolyticus* isolates based on their antimicrobial susceptibility profiles and location (moderate- and high-salinity sites) during the relaying trials. The PFGE profiles of *V. parahaemolyticus* were genetically diverse, and no genetic relationship was noted among the sampling sites, antimicrobial susceptibility test results, pathogenicity, and O serogroups. A similar trend was also observed for the PFGE profiles of *V. vulnificus* isolates. Our results are similar to the results of a study conducted by Lewis et al. (25) in which *V. vulnificus* isolates recovered pre- and postdepuration were analyzed using the amplified fragment length polymorphism (AFLP) technique. All recovered isolates clustered at 40% or higher similarity, which indicates the high intraspecific diversity of this species. Previous investigators (4, 46) also found that the PFGE analysis demonstrated high heterogeneity among the *V. parahaemolyticus* isolated from seafood.

Conclusion. This study provides baseline data on the phenotypic and genotypic characteristics of *Vibrio* spp. in oysters and the effect of relaying to high-salinity sites on these characteristics. Among the PCR-confirmed *V. vulnificus* and *V. parahaemolyticus* isolates recovered from oyster samples, 20.9% of *V. vulnificus* isolates were positive for the *vcgC* gene, while among *V. parahaemolyticus* isolates, only 0.7% and 2.2% were positive for the *tdh* and *trh* genes, respectively, and none of the tested isolates were positive for both genes. Analysis of the antibiotic resistance profiles of *V. vulnificus* and *V. parahaemolyticus* isolated from oysters indicated that *V. vulnificus* isolates possessed a higher rate of resistance or intermediate resistance, as well as a higher rate of multiple resistance to almost all tested antibiotics, including those recommended by the CDC for treating *Vibrio* infections. Also, *V. parahaemolyticus* showed high MICs for some of the *Vibrio* infection treatment antibiotics. No significant effect of the relaying process was observed on the antimicrobial resistance profiles or the presence of virulence genes of either *V. vulnificus* or *V. parahaemolyticus* isolates. Molecular characterization of the selected *V. vulnificus* and *V. parahaemolyticus* isolates showed a high genetic diversity, even among the isolates obtained from the same site and collection day.

TABLE 5 Concentration ranges of selected antibiotics tested for *V. vulnificus* and *V. parahaemolyticus* according to CLSI guidelines

Antibiotic (abbreviation)	Concn (μl/ml)
Amikacin (AMI)	4–64
Amoxicillin-clavulanic acid (AUG2)	2–16/1–8
Ampicillin (AMP)	2–16
Ampicillin-sulbactam (A/S2)	2–16/1–8
Cefepime (FEP)	0.5–16
Cefotaxime (FOT)	0.03–2
Cefoxitin (FOX)	4–32
Ceftazidime (TAZ)	4–32
Ceftriaxone (AXO)	0.5–16
Cephalothin (CEP)	2–16
Chloramphenicol (CHL)	2–16
Ciprofloxacin (CIP)	0.25–2
Doxycycline (DOX)	0.5–8
Imipenem (IMI)	1–8
Levofloxacin (LEVO)	0.5–4
Meropenem (MERO)	0.25–8
Piperacillin (PIP)	1–64
Piperacillin-tazobactam (P/T4)	1–32/4–4
Tetracycline (TET)	0.5–8
Trimethoprim-sulfamethoxazole (SXT)	2–4/38–76

MATERIALS AND METHODS

This study was conducted as part of an effort to examine the influence of high-salinity relay on *Vibrio* abundance in Chesapeake Bay and Maryland Coastal Bay oysters (*Crassostrea virginica*). Oysters were purchased from a commercial aquaculture facility (the salinity of the original harvest site was 10.3 to 16.3 ppt), and the harvested oysters were transported to the relaying sites (Pocomoke Sound, MD [moderate salinity, 12 to 18.7 ppt], and Chincoteague Bay, MD [high salinity, 28 to 32.5 ppt]). One hundred twenty oysters were placed in 2 ft by 3 ft oyster cages (3 cages at each site) and placed overboard. At each selected time interval (days 0, 7, 14, and 21), 3 composites were collected from each relaying site. Collected oysters were analyzed for presumptive *V. parahaemolyticus* and *V. vulnificus*, and abundances were determined using the alkaline peptone water (APW) 3-tube MPN series (26).

Isolation of *V. vulnificus* and *V. parahaemolyticus* from oyster samples. To isolate *V. parahaemolyticus* colonies, from the top 1 cm of turbid (positive) APW tubes, a 3-mm loopful was streaked onto thiosulfate-citrate-bile-salt-sucrose (TCBS) agar, and for *V. vulnificus* isolation, turbid APW tubes were streaked on modified cellobiose polymyxin B-colistin (mCPC) agar. The inoculated plates were incubated at 35°C for TCBS and at 39°C for mCPC. Three to five positive colonies were picked from each MPN dilution (10^{-1} to 10^{-6}) and frozen for further examination (26).

Approximately, a total of 400 colonies of each *V. parahaemolyticus* and *V. vulnificus* were isolated during the 3 relaying trials. In this study, we evaluated the pathogenicity, antimicrobial susceptibility, and genetic profiles of all retrieved viable *V. parahaemolyticus* and *V. vulnificus* colonies, as some of the isolates lost their viability during storage.

PCR assay for the virulence genes. A total of 296 *V. parahaemolyticus* and 94 *V. vulnificus* PCR-confirmed samples (74% and 24%, respectively) of the total samples isolated during relaying trials (interval days, 0, 7, 14, and 21) (26) were subjected to further testing for the presence of pathogenic (*tdh*$^+$ and/or *trh*$^+$) genes using a real-time PCR *V. parahaemolyticus* multiplex assay (47). Real-time PCR was also used for the detection of virulence-correlated gene type C (*vcgC*) in *V. vulnificus* (41). Twenty-three microliters of a master mix consisting of 1× PCR amplification buffer (Invitrogen, Carlsbad, CA), 5.0 mM $MgCl_2$ (Invitrogen), 400 nM each of the deoxynucleoside triphosphates (Roche, Indianapolis, IN), 200 nM (each) the *trh* and *tdh* forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 75 nM (each) the *tlh* forward and reverse primers (Integrated DNA Technologies), 150 nM probe for *tlh* (Integrated DNA Technologies), 75 nM probe for *tdh* and *trh* (Applied Biosystems, Foster City, CA), and 2.25 U platinum *Taq* DNA polymerase (Invitrogen) was used. The remainder of the reaction mixture volume consisted of 2 μl of the template (boiled sample). Real-time PCR thermal cycling was conducted using a SmartCycler II system from Cepheid (Sunnyvale, CA). For the *vcgC* multiplex assay, the assay solution was composed of 20 μl of master mix and 5 μl of template. The following analysis settings were adjusted: "manual threshold fluorescence units" to 15 for the 6-carboxyfluorescein (FAM [*tdh*]), TET (*trh*), TxRed (*tlh*, total *V. parahaemolyticus*), and Cy5 (internal control) channels (26). Positive (for each targeted gene) and negative (PCR water) controls were included for each run.

Antimicrobial susceptibility test. A total of 150 (38% of the collected colonies) *V. parahaemolyticus* (n = 92 [2 *tdh*$^+$ and 2 *trh*$^+$]) and *V. vulnificus* (n = 58 [9 *vcgC*$^+$]) isolates were collected from oysters during the moderate- and high-salinity relaying trials (26). All isolates were tested for their susceptibility to 20 different antibiotics, chosen by clinical usage for these pathogens and aquaculture practices (Table 5), using the Sensititre microbroth dilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (48). The MIC was determined as the lowest concentration of an

antimicrobial that completely inhibited the growth of bacteria. Multidrug resistance was defined as an absence of susceptibility to two or more classes of antibiotics (40).

Identification of the somatic (O) serotypes of *V. parahaemolyticus* isolates. To determine the predominant O serotypes of the 92 viable and confirmed *V. parahaemolyticus* isolates recovered from oysters, the slide agglutination test was conducted using commercially available antisera that included 13 different O antigens, as described by the manufacturer (Denka Seiken Co., Ltd.).

PFGE. To determine the effect of the relaying process on the genetic profile of the *Vibrio* isolates, *V. parahaemolyticus* ($n = 64$ [2 *tdh*$^+$ and 2 *trh*$^+$]) and *V. vulnificus* ($n = 58$ [9 *vcgC*$^+$]) isolates with different antibiotic susceptibility profiles were selected. Isolates were inoculated onto tryptic soy agar (TSA) plus 1% NaCl and incubated overnight at 35°C. Plug preparation and PFGE were performed using the SfiI restriction enzyme, as described in the CDC PulseNet protocol for *V. parahaemolyticus* and *V. vulnificus* (49, 50). The gel was stained with ethidium bromide, and DNA bands were visualized with a UV light.

Statistical analysis. Descriptive and inferential statistics were used to compare the frequencies of isolates testing positive for virulence genes and exhibiting intermediate resistance or resistance to antibiotics by site and/or day of collection during the relaying study. A Kruskal-Wallis one-way analysis of variance (ANOVA) was used to assess the significance of observed differences in the frequencies of antibiotic resistance of *V. vulnificus* and *V. parahaemolyticus* isolates collected from relaying sites with different salinities.

PFGE fingerprints were analyzed with the BioNumerics software 7.0 (Applied Maths) using standard band calling methods. Dendrograms were constructed using the Dice correlation, with 1.5% optimization and 1.5% tolerance, and the unweighted pair group method using average linkages (UPGMA) (50). Identification of clusters within a dendrogram was based on a 76% similarity. BioNumerics software version 7.0 (Applied Maths, Belgium) was used to analyze the DNA bands. Clustering was performed using UPGMA and the Dice correlation coefficient, with a position tolerance of 1.5%.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01790-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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