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FOOD MICROBIOLOGY

Antimicrobial Resistance, Virulence Factors, and Genetic Profiles of *Vibrio parahaemolyticus* from Seafood

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ABSTRACT *Vibrio parahaemolyticus* is a widespread bacterium in the marine environment and is responsible for gastroenteritis in humans. Foodborne infections are mainly associated with the consumption of contaminated raw or undercooked fish and shellfish. The aim of this study was to determine the antimicrobial resistance, virulence factors, and genetic profiles of *V. parahaemolyticus* isolates from seafood originating from different countries. A total of 104 (17.5%) isolates were recovered from 595 analyzed samples. The isolates were tested for the presence of the *tdh* and *trh* genes, involved in the pathogenesis of *V. parahaemolyticus* infections in humans, and these genes were detected in 3 (2.9%) and 11 (10.6%) isolates, respectively. The *trh*-positive isolates also possessed the *ure* gene, which is responsible for urease production. Moreover, the activity of protease A was identified in all *V. parahaemolyticus* strains. Antimicrobial resistance revealed that most isolates were resistant to ampicillin (75.0%) and streptomycin (68.3%), whereas all strains were sensitive to chloramphenicol and tetracyclines. Most of the isolates (55.8%) showed resistance against two classes of antimicrobials, mainly to ampicillin and streptomycin (46.2%). Only one isolate displayed a multiresistant pattern. Genotypic analysis of *V. parahaemolyticus* revealed a high degree of diversity among the isolates tested. The pulsed-field gel electrophoresis (PFGE) method distinguished 73 clonal groups, and the most numerous group consisted of 7 strains. Sequencing by the multilocus sequence typing (MLST) method showed 76 sequence types (STs), of which ST481 and ST1361 were most frequently identified. In addition, 51 (67.1%) new sequence types were discovered and added to the PubMLST international database.

IMPORTANCE The presence of *V. parahaemolyticus* in seafood may pose a risk for consumers, especially in countries where shellfish are eaten raw. In recent years, a significant increase of food poisoning caused by these bacteria has been also observed in Europe. Our results highlight the high level of *V. parahaemolyticus* contamination of seafood, along with the isolates being potentially pathogenic for humans. However, the first-line antimicrobials, such as tetracyclines and fluoroquinolones, remained highly effective against *V. parahaemolyticus*. The monitoring of antimicrobial resistance of isolates is important to ensure the high efficacy in the treatment of human infections. Most of *V. parahaemolyticus* strains possessed new sequence types (STs), which showed the high genetic diversity of the isolates tested.

KEYWORDS *Vibrio parahaemolyticus*, virulence factors, antimicrobial resistance, MLST, PFGE, genetic diversity

Vibrio parahaemolyticus is a marine microorganism recognized as an important cause of gastroenteritis in humans (1, 2). These bacteria naturally occur in warm seawaters along the coasts of many continents, and their highest level is found in the summer months. However, in recent years, increasing water temperature in seas and oceans has been observed as a result of global warming and may lead to the appearance of this pathogen in seawater, where it has not been previously present (2, 3). Human infection

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occurs usually after eating of contaminated food, especially raw or undercooked fish, crustaceans, and molluscs. Pathogenic *V. parahaemolyticus* bacteria mainly cause gastrointestinal disorders characterized by watery diarrhea, nausea, and abdominal cramps, but in severe cases, septicemia and generalized disease may develop, which sometimes lead to death (4). In Asian countries and the United States, *V. parahaemolyticus* has been one of the most common causes of food poisoning in humans (1, 5, 6). In Europe, the number of *V. parahaemolyticus* infections is growing year by year, mainly due to climate warming and changes in eating habits, i.e., increasing consumption of seafood (2).

Most of the *V. parahaemolyticus* strains isolated from the environment or food have no pathogenic potential, whereas clinical isolates usually possess virulence factors, such as thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH) (1). Both hemolysins, encoded by the *tdh* and *trh* genes, respectively, are the most important virulence markers associated with hemolytic, enterotoxic, and cytotoxic activities in the host cell (4, 7). Moreover, *trh*-positive *V. parahaemolyticus* strains almost always produce urease, which plays a significant role in the colonization of intestinal epithelial cells as well as in the activation of inflammatory cytokines (4, 8). However, *V. parahaemolyticus* strains that do not produce any hemolysins may possess other virulence factors important in gastroenteric infections (4, 9).

The vast majority of *V. parahaemolyticus* infections are self-limiting, and antimicrobial therapy is rarely required. The antimicrobials of choice for the treatment of severe infection cases are cephalosporins, tetracyclines, quinolones, and fluoroquinolones (10, 11). However, in recent years, many antimicrobial-resistant *V. parahaemolyticus* strains have emerged into the environment due to the excessive use of antibiotics and other chemotherapeutic agents in humans, agriculture, and aquaculture (10, 12). A determination of the antimicrobial resistance profile among *V. parahaemolyticus* strains may be used for monitoring changes in the sensitivity of the bacteria to antibiotics, especially those of the first-line choices in the treatment of human infections (13).

Several molecular typing methods have been developed for the differentiation of *V. parahaemolyticus* isolates, tracking the source of infection and detection of geographic distribution of virulent strains (14). Pulsed-field gel electrophoresis (PFGE) is a highly discriminatory technique used to determine the relatedness of clinical and environmental isolates and is still considered to be the gold standard for *V. parahaemolyticus* typing (15). Newer genotyping methods applied in epidemiological investigation, including multilocus sequence typing (MLST), are based on the sequencing of genes or whole genomes (14, 16). MLST allows the detection of slowly progressing sequence changes in the *V. parahaemolyticus* genome and may be used in monitoring the spread of pathogenic strains (14, 16).

The aim of the present study was to characterize *V. parahaemolyticus* isolates from seafood for the presence of virulence factors, antimicrobial resistance, and molecular diversity using PFGE and MLST methods.

RESULTS

Prevalence of *V. parahaemolyticus*. A total 113 of 595 (19.0%) fish and shellfish samples tested were positive for *V. parahaemolyticus* as a result of biochemical identification, whereas PCR for the species-specific *toxR* and *tlh* genes confirmed the presence of these bacteria in 104 (92.0%) samples (Table 1). Most of the isolates were recovered from bivalve molluscs (92 out of 104 isolates [88.5%]), including clams (50 [54.4%]), mussels (25 [27.2%]), oysters (12 [13.0%]), and scallops (5 [5.4%]). The remaining 12 (11.5%) strains were obtained from fish. Regarding the geographical origin, *V. parahaemolyticus* was most commonly isolated from samples from the Netherlands (45 isolates [43.3%]) and Italy (34 [32.7%]), but some strains were also from Norway (13 [12.4%]), France (6 [5.8%]), and other countries (6 [5.8%]).

Identification of virulence factors. The *tdh* gene was identified only in 3 of 104 (2.9%) *V. parahaemolyticus* isolates analyzed, and all of them were recovered from Manila clams originating from Italy. The *trh* marker was detected in 11 (10.6%) isolates, of which 9 isolates were obtained from shellfish, especially from clams (7 strains) and

TABLE 1 Sources of *V. parahaemolyticus* strains used in the present study

Sample type tested	No. (%) of samples	
	Tested	Positive
Shellfish		
Clams		
Manila clams	122	50
Razor clams	24	0
Hard clams	7	0
Cockle	6	0
Amandes	4	0
Mussels	150	25
Oysters	129	12
Scallops	53	5
Total	495 (83.2)	92 (15.5)
Fish		
Perch	37	5
Cod	16	0
Salmon	15	2
Flounder	13	1
Herring	10	2
Ocean perch	7	1
Angler fish	2	1
Total	100 (16.8)	12 (2.0)
All samples	595 (100.0)	104 (17.5)

fish (2 isolates). None of the tested isolates had both the *tdh* and *trh* genes. Moreover, all *trh*-positive strains possessed the *ure* gene and were able to produce urease *in vitro*. In addition, urease activity was revealed in one isolate from a fish that was *trh* negative. All *V. parahaemolyticus* isolates also showed protease A activity.

Antimicrobial resistance. The antimicrobial resistance profiles of 64 *V. parahaemolyticus* isolates included in the present study were described previously (17). The analysis of the results for all 104 isolates revealed that 91 (87.5%) of them were resistant to at least one antimicrobial agent used in the study, and the remaining 13 (12.5%) strains were susceptible to all antimicrobials. The majority of the *V. parahaemolyticus* strains were resistant to ampicillin (78 [75.0%]) and streptomycin (71 [68.3%]), whereas resistance to gentamicin was found in 13 (12.5%) of the strains tested. On the other hand, only one isolate was resistant to ciprofloxacin, and all *V. parahaemolyticus* isolates were susceptible to tetracycline and chloramphenicol. Differences in antimicrobial resistance were observed among strains of various sources and geographical origins (Fig. 1). Most isolates resistant to ampicillin (40 [80.0%]) and streptomycin (40 [80.0%]) were recovered from clams. Ampicillin-resistant strains mainly originated from fish and shellfish from Italy (28 [82.3%]) and from the Netherlands (33 [73.3%]), whereas *V. parahaemolyticus* strains resistant to streptomycin were mainly recovered from Italian samples (29 [85.3%]). Isolates resistant to gentamicin were from mussels (1 isolate [4.0%]), oysters (1 isolate [8.3%]), and clams (11 [22.0%]), which originated from Italy, the Netherlands, and Norway. The only isolate resistant to ciprofloxacin was from Italian clams. It was noticed that the prevalences of streptomycin-resistant isolates from clams (40 out of 50 [80.0%]) and oysters (5 out of 12 [41.7%]) were statistically significant ($P < 0.05$). However, the analysis of the relationship between the country of origin and antimicrobial resistance did not reveal any differences ($P > 0.05$). Most of *V. parahaemolyticus* (58 [55.8%]) isolates showed resistance against two classes of antimicrobials, mainly to ampicillin and streptomycin (48 [46.2%]). Some strains were resistant to ampicillin, streptomycin, and gentamicin (10 [9.6%]). Only one isolate was resistant to three antimicrobial classes (ampicillin, streptomycin, and ciprofloxacin).

MLST. MLST analysis showed high molecular diversity among 104 *V. parahaemolyticus* isolates tested in the study. A total of 76 different sequence types (STs) were identified, including 17 STs with at least two isolates and 59 STs containing single

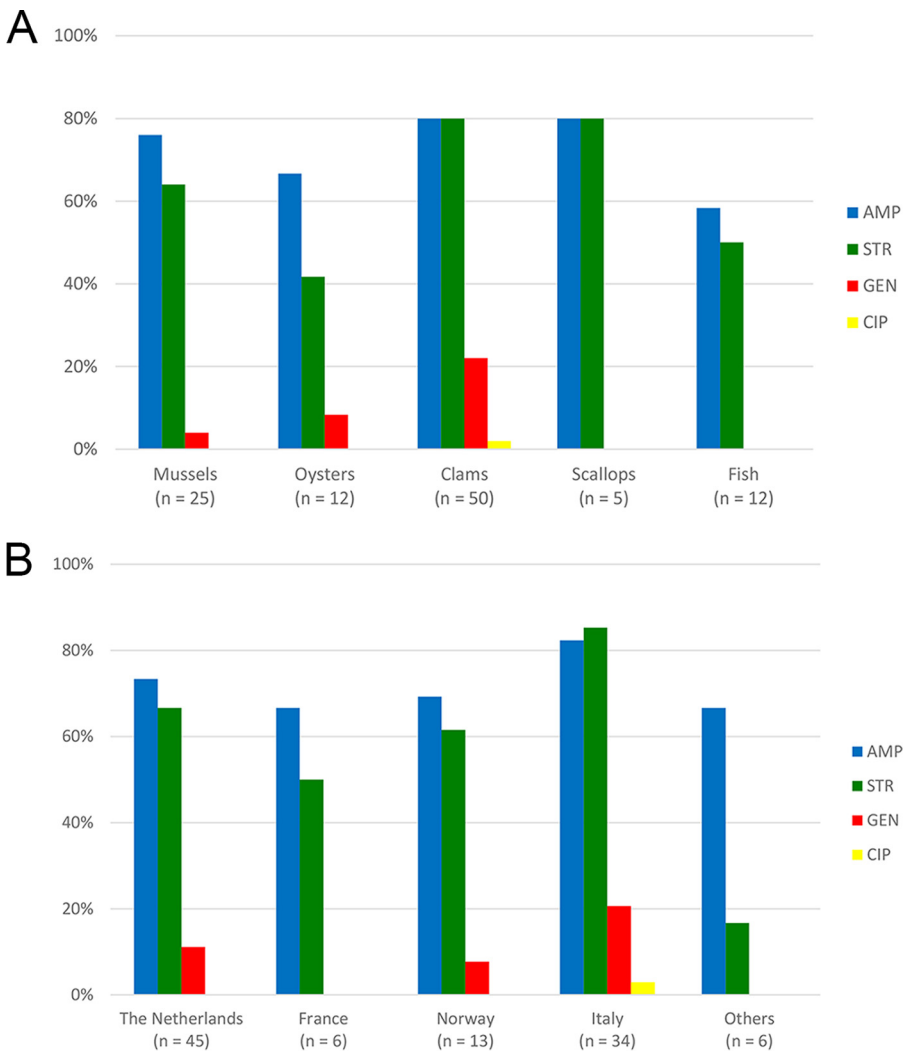

FIG 1 Percentages of 104 *V. parahaemolyticus* isolates resistant to antimicrobials in relation to source (A) and origin (B) of samples. AMP, ampicillin; STR, streptomycin; GEN, gentamicin; CIP, ciprofloxacin.

strains. Furthermore, 51 (67.1%) novel STs were discovered and added to the international PubMLST/*V. parahaemolyticus* database (http://pubmlst.org/vparahaemolyticus) (Table 2). The two most frequent sequence types, ST481 and ST1361, possessed 5 and 4 isolates, respectively. Strains with ST481 were closely related (similarity above 80%) to the isolates of ST6 and ST1359 (difference only in the *dtdS* gene) and to the strain from ST1389 (which possessed a new allele for the *dnaE* gene). The minimum spanning tree based on the allele numbers and geographical origins of the isolates did not reveal a clear clustering connected with the origin of the strains (Fig. 2).

PFGE. PFGE investigation showed 73 different molecular patterns, with 20 groups containing at least two isolates and the remaining 53 profiles covering only one strain (Table 2). The largest cluster group (PFGE 60) included 7 strains, mainly of clams (6 isolates) and scallops (1 isolate) originating from different countries. The majority of these isolates were classified to ST481 (5 strains) and were resistant to ampicillin and streptomycin. Most isolates with the same PFGE profile had similar ST and antibiotic resistance patterns and were mostly isolated from the same source. It was also found that *V. parahaemolyticus* isolates of the PFGE clonal groups 6, 32, 58, 60, and 61 possessed the same macrorestriction profiles but other STs. On the other hand, the isolates with the same sequence types ST49, ST445, and ST1361 had different PFGE profiles.

TABLE 2 PFGE profiles in relation to sequence types and AMR patterns of *V. parahaemolyticus* strains originating from various sources and geographical areas[a]

PFGE profile	No. of isolates	Source (no.) of isolates[b]	Country (no.) of origin[c]	AMR pattern (no.)	ST (no.)[d]
1	2	M (2)	NO (2)	AMP (1), AMP-STR (1)	**ST1371** (2)
2	1	O	NL	AMP-STR-GEN	**ST1407**
3	2	M (1), O (1)	FR (1), NL (1)	AMP (2)	**ST1366** (2)
4	1	C	NL	AMP	**ST1417**
5	1	M	NO		**ST1388**
6	2	C (1), S (1)	IT (1), NO (1)	AMP-STR (2)	**ST1356** (1), **ST1382** (1)
7	1	C	IT	AMP-STR	**ST1368**
8	1	F	NL	AMP-STR	**ST1385**
9	1	M	NO	AMP	**ST1369**
10	1	M	NO	AMP-STR	**ST1379**
11	1	M	NL	AMP-STR	**ST1374**
12	1	C	IT	AMP-STR	ST162
13	1	M	NL	AMP	ST57
14	1	C	IT	AMP-STR	**ST1380**
15	1	C	NL	AMP	**ST1383**
16	1	O	NL	AMP-STR	**ST1370**
17	2	O (2)	NL (2)		**ST1411** (2)
18	1	C	NL	AMP-STR-GEN	**ST1419**
19	1	C	NL	AMP	ST445
20	1	M	NO	STR-GEN	ST1320
21	3	M (3)	NL (3)	AMP-STR (3)	**ST1372** (3)
22	2	M (1), S (1)	IT (1), JP (1)		**ST1386** (2)
23	1	O	NL	AMP	ST49
24	2	M (2)	DK (1), NL (1)	AMP-STR (2)	ST49 (2)
25	1	C	NL	AMP-STR	**ST1375**
26	1	C	IT	AMP-STR	ST400
27	1	F	NL	AMP-STR	**ST1414**
28	1	O	NL	AMP	**ST1373**
29	2	M (2)	NL (2)	STR (1), AMP-STR (1)	ST347 (2)
30	1	C	IT	AMP	**ST1409**
31	1	C	FR	AMP	**ST1363**
32	2	M (2)	NL (2)	AMP (1), AMP-STR (1)	ST445 (1), ST774 (1)
33	2	O (2)	NL (2)	AMP (1), STR (1)	ST1195 (2)
34	1	C	FR	AMP-STR	ST1159
35	1	C	NL	AMP-STR	ST1285
36	1	C	FR		**ST1416**
37	2	C (2)	NL (2)		ST810 (2)
38	1	M	NL	AMP-STR	ST767
39	1	F	DE		**ST1413**
40	1	F	NO		ST73
41	1	F	NL	AMP-STR	ST64
42	3	C (3)	IT (2), NL (1)	AMP-STR (1), AMP-STR-GEN (2)	**ST1358** (3)
43	1	C	IT	GEN	**ST1387**
44	1	S	NL	AMP-STR	**ST1381**
45	1	C	IT	AMP-STR	**ST1410**
46	1	C	NL	AMP-STR	ST135
47	3	F (3)	LK (1), TR (2)	AMP (3)	**ST1390** (3)
48	1	F	NO		ST996
49	1	O	NL	AMP-STR	**ST1406**
50	1	C	IT	AMP-STR-GEN	**ST1415**
51	3	C (2), F (1)	FR (1), IT (2)	AMP-STR (1), AMP (2)	**ST1362** (3)
52	1	C	IT	AMP-STR	**ST1365**
53	1	C	IT	AMP-STR-CIP	**ST1367**
54	1	C	IT	AMP-STR-GEN	**ST1357**
55	1	C	IT	AMP-STR	**ST1405**
56	1	C	IT	AMP-STR-GEN	**ST1377**
57	3	C (1), F (2)	IT (2), NL (1)	AMP-STR (2), STR (1)	**ST1384** (3)
58	2	C (2)	NL (2)	AMP-STR-GEN (2)	ST6 (1), **ST1376** (1)
59	1	C	IT	AMP-STR	**ST1412**
60	7	C (6), S (1)	FR (1), IT (4) NL (1), NO (1)	AMP-STR (3), AMP (2), STR (1), STR-GEN (1)	ST481 (5), **ST1359** (1), **ST1389** (1)
61	2	C (1), M (1)	IT (1), NO (1)	AMP-STR (2)	ST564 (1), ST1290 (1)
62	1	C	NL	AMP-STR	**ST1418**
63	1	C	IT	AMP-STR	ST490

(Continued on next page)

TABLE 2 (Continued)

PFGE profile	No. of isolates	Source (no.) of isolates[b]	Country (no.) of origin[c]	AMR pattern (no.)	ST (no.)[d]
64	1	O	NL	AMP	**ST1404**
65	1	C	IT	AMP-STR-GEN	**ST1408**
66	1	O	NL	AMP	**ST1360**
67	3	C (1), M (2)	IT (1), NL (2)	AMP (2), AMP-STR (1)	**ST1361** (3)
68	1	M	NL		**ST1361**
69	1	S	NO	AMP-STR	ST1267
70	2	C (1), M (1)	IT (1), NO (1)	AMP-STR (2)	ST773 (2)
71	1	C	IT	AMP-STR	**ST1355**
72	1	C	IT		**ST1364**
73	1	C	IT	AMP-STR	ST1262

[a]AMR, antimicrobial resistance.
[b]C, clams; O, oysters; M, mussels; S, scallops; F, fish.
[c]NL, the Netherlands; IT, Italy; NO, Norway; FR, France; DK, Denmark; JP, Japan; DE, Germany; TR, Turkey; LK, Sri Lanka.
[d]Novel sequence types (STs) are marked in bold.

DISCUSSION

The present results represent a comprehensive study on the characterization of *V. parahaemolyticus* isolates toward the presence of virulence factors, antimicrobial resistance, and genetic relatedness.

The *tdh* and *trh* hemolysin markers, which play a significant role in the pathogenesis of infection in humans, were identified only in 3 (2.9%) and 11 (10.6%) of 104 isolates, respectively. In the study by Bilung et al. (18), the *tdh* gene was also detected at a lower level (3.2%) than the *trh* gene (17.7%). Similar results were also obtained by Ottaviani

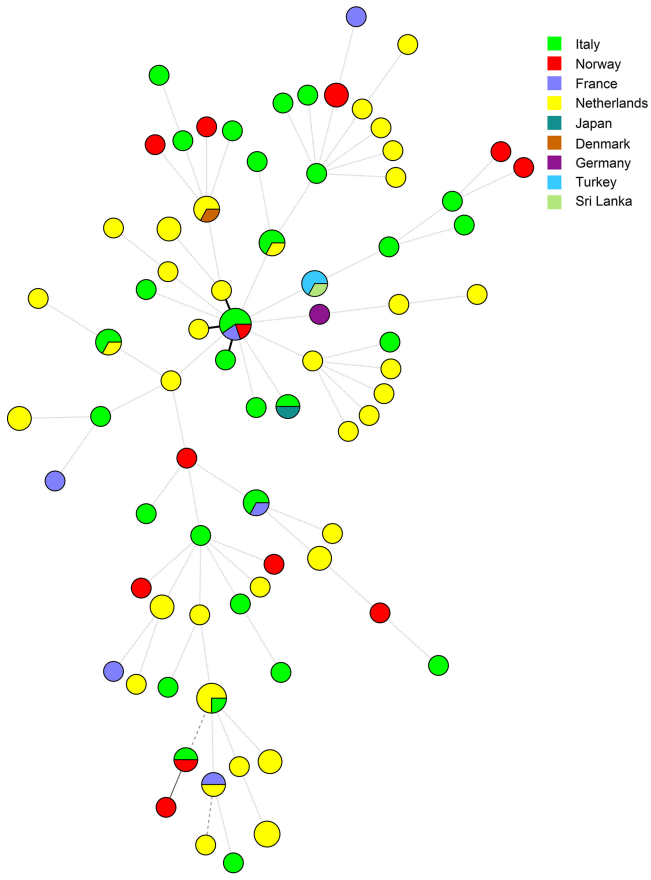

FIG 2 Minimum spanning tree of *V. parahaemolyticus* strains based on the allelic profiles. Colors indicate the geographical origins of isolates. Size of circles represents number of isolates with the same ST, and thickness of the branches indicates the degree of similarity among *V. parahaemolyticus* strains tested.

et al. (19). On the other hand, a higher prevalence of the *tdh* gene than the *trh* marker was observed in *V. parahaemolyticus* in Spain and in Thailand (20, 21). However, the distribution of *tdh*- and/or *trh*-positive strains may vary depending on the geographical origin, sample source, and the method of their detection. Furthermore, all *trh*-positive isolates identified in the present investigation possessed the *ure* gene, which is due to the presence of both genes in the same chromosomal pathogenicity island (8). Our results confirmed data reported by Sujeewa et al. (22) that not all isolates capable of producing urease were *trh* positive. Moreover, in the present investigation, another potential pathogenic factor, protease A, was found in 100% of the isolates tested, similar to the results in the study of Ottaviani et al. (9). This confirms the presence of other virulence factors, besides both hemolysins in *V. parahaemolyticus*, which play a significant role in pathogenicity.

In the current investigation, 75.0% and 68.3% of the isolates showed resistance to ampicillin and streptomycin, respectively, and these results were comparable to data obtained in other countries (10, 23, 24). The majority of *V. parahaemolyticus* strains resistant to both antibiotics were isolated from clams, mainly originating from Italy, which was similar to the findings in previous reports by Yu et al. (5), who found that 96.7% and 68.0% of *V. parahaemolyticus* strains of clam and oyster origin, respectively, were resistant to ampicillin and streptomycin. The highest resistance to ampicillin (100.0%) was previously observed among the isolates from Italian samples, whereas the percentage of streptomycin-resistant strains from this country was lower (32.2%) than that in the current study (23). The high resistance to β-lactams and aminoglycosides observed among *V. parahaemolyticus* strains may be due to extensive use of these antimicrobials in treatment, agriculture, and aquaculture during recent decades (10, 12). Moreover, in water environments, different microorganisms, including *V. parahaemolyticus*, are able to exchange their genetic determinants, which may also be the cause of increasing resistance to antibiotics (4, 23). Additionally, 46.2% of the isolates showed resistance to ampicillin (AMP) and streptomycin (STR), 9.6% to AMP, STR, and gentamicin (GEN), and only one isolate recovered from clams from Italy was resistant to ampicillin, streptomycin, and ciprofloxacin. In other investigations, most of the isolates (60 out of 76 [78.9%]) were resistant to two or more antimicrobial agents (25), whereas relatively low percentages (3.7% and 10.3%) of strains resistant to two and three classes of antimicrobials, respectively, were found in Italy (23). However, a comparison of data from various studies is often difficult due to different samples' origins, time and methods of isolate collection, and laboratory analysis. Furthermore, the present results confirmed those of previous reports from Europe, the United States, and Asian countries that *V. parahaemolyticus* strains were generally susceptible to antimicrobial agents used in the treatment of human infections (11, 21, 23). However, there is also a study showing a high prevalence of strains resistant to ciprofloxacin (46.9%), chloramphenicol (36.7%), and tetracycline (81.6%) (26).

Molecular typing revealed a high degree of diversity of the strains tested. The MLST method showed 76 STs, of which 51 (67.1%) STs were newly identified. Similar results were reported by Urmersbach et al. (27), where 130 *V. parahaemolyticus* strains obtained from a marine environment were classified into 82 STs, and 82.9% of them were described as new. Rahman et al. (28) also identified 63 unique STs in the isolates tested, and 49 (77.8%) STs were not described in the database. On the other hand, a lower percentage of isolates with new sequence types was also noted (29). Our findings and data from other countries indicated a high number of new alleles and STs in *V. parahaemolyticus* which have not been included into database. However, molecular information on the isolates collected in PubMLST are from clinical samples from the United States and Asia, whereas the isolates used in the present study were mainly originated from the European countries.

Based on the PFGE analysis, 73 clonal groups were identified among the isolates tested, with an overall similarity of 43.5%. Other authors indicated a closer genetic correlation between *V. parahaemolyticus* strains isolated in Europe or Japan, which were 63.4% and 71.0%, respectively (15, 30). However, there are also reports of a much

lower level of similarity (28.4%) among isolates tested (31). The above-mentioned results are difficult to compare due to various sources of origin and different periods of *V. parahaemolyticus* isolation. It was also found that the largest PFGE 60 group, with 7 strains recovered mainly from clams in Italy, was resistant to ampicillin and streptomycin and belonged to ST481. This sequence type was previously identified in environmental isolates from Germany and Italy, as well as in clinical strains from China (27, 28) (http://pubmlst.org/vparahaemolyticus). In the present study, ST481 was closely related to new ST1359 and ST1389 detected in *V. parahaemolyticus* from clams originating from Italy and the Netherlands, respectively. Moreover, ST6 with the *trh* and *ureR* genes identified in the isolates from the Netherlands was genetically similar to strains of ST481. This sequence type was previously described in environmental isolates from Chile, Norway, and Italy (16, 32) (http://pubmlst.org/vparahaemolyticus). It may suggest a worldwide spread of some STs in *V. parahaemolyticus*, regardless of geographical location and environmental conditions.

In conclusion, the results of the present study indicate that *V. parahaemolyticus* may occur in seafood available in Poland, especially during warmer months. Some of these strains had pathogenic properties due to the presence of virulence factors. Most of the *V. parahaemolyticus* strains possessed new sequence types, which indicates a high diversity of the strains. Several isolates were resistant to ampicillin and streptomycin as well as to more than one class of antibiotics. Therefore, these bacteria may pose a serious threat to consumer health.

MATERIALS AND METHODS

Sources and identification of *V. parahaemolyticus*. *Vibrio parahaemolyticus* strains were isolated during 2009 to 2015 from different species of raw shellfish ($n = 495$) and marine fish ($n = 100$) available on the Polish market but originating from various countries, such as the Netherlands ($n = 277$), Norway ($n = 127$), Italy ($n = 91$), France ($n = 66$), Turkey ($n = 12$), Poland ($n = 10$), Denmark ($n = 4$), Spain ($n = 4$), Germany ($n = 2$), and Sri Lanka ($n = 2$). All samples were analyzed according to the ISO 21872-1 standard (33) and then confirmed by PCRs for the species-specific *tlh* and *toxR* genes, as described previously (34, 35). Among all 104 *V. parahaemolyticus* isolates, 64 isolates were already described in a previous study on the prevalence and antimicrobial resistance (17). Detailed information on the number of *V. parahaemolyticus* isolates used in the current investigation is presented in Table 1.

Determination of virulence factors. *Vibrio parahaemolyticus* isolates were investigated for the presence of the *tdh*, *trh*, and *ureR* genes using PCRs, as described previously (36, 37). The amplification products were visualized on 1.5% agarose gels (Sigma-Aldrich, USA) by staining with ethidium bromide (Sigma-Aldrich) and photographed using the Gel Doc 2000 documentation system (Bio-Rad, USA). Expression of the *ure* gene was determined by capacity of urease production on Christensen's urea agar with 1% NaCl (9). Protease A activity was examined on a nutrient agar with 1.5% skimmed milk and 1% NaCl (38).

Determination of antimicrobial resistance. A broth microdilution method was used to establish the MICs of *V. parahaemolyticus* to antimicrobials selected according to the Clinical and Laboratory Standards Institute (CLSI) guideline (39), as described previously (17). The Sensititre custom susceptibility EUMVS2 plates (Trek Diagnostic Systems, UK) contained the following antibacterial agents (dilution range) were used: ampicillin (AMP, 0.5 to 32 mg/liter), ciprofloxacin (CIP, 0.03 to 8 mg/liter), streptomycin (STR, 2 to 128 mg/liter), gentamicin (GEN, 0.25 to 32 mg/liter), tetracycline (TET, 1 to 64 mg/liter), and chloramphenicol (CHL, 2 to 64 mg/liter). The cutoff values used for the interpretation of the MIC results were in accordance with the CLSI guideline (39), except streptomycin, for which the breakpoint has been described elsewhere (11, 24).

MLST analysis. Multilocus sequence typing was performed by the sequence analysis of seven housekeeping genes (*recA*, *gyrB*, *dnaE*, *dtdS*, *pntA*, *pyrC*, and *tnaA*) according to the protocol available on the PubMLST website (http://pubmlst.org/vparahaemolyticus). The received nucleotide sequences for each locus were analyzed with BioNumerics software version 7.6 (Applied Maths, Belgium) and compared to the already-published sequences on the PubMLST website. Sequence types (STs) were determined on the basis of the obtained seven-digit allelic profiles. New alleles were submitted to the *V. parahaemolyticus* database in PubMLST. A phylogenetic tree was created using the unweighted pair group method using average linkages (UPGMA) and the minimum spanning tree (MST) methods (40, 41).

PFGE typing. Pulsed-field gel electrophoresis was done according to the CDC PulseNet protocol (42). DNA was digested with 40 U of SfiI enzyme (Thermo Fisher Scientific, USA) at 50°C for 4 h. The separation of restriction fragments was performed in 1% SeaKem gold agarose (Lonza, USA) gels in 0.5× Tris-borate-EDTA (TBE) buffer (Sigma-Aldrich) using the CHEF-DR III system (Bio-Rad), with the following parameters: initial time, 10 s; final time, 35 s for 18 to 19 h at 6 V/cm and 14°C. The gels were stained in ethidium bromide (5 μg/ml; Sigma-Aldrich) for 15 min, and the DNA banding patterns were visualized with the Gel Doc 2000 system. *Salmonella enterica* serovar Braenderup H9812 was used as the molecular weight standard (42, 43). The macrorestriction profiles were analyzed by the BioNumerics software, and

the dendrogram was generated by the UPGMA with the Dice correlation coefficient and a position tolerance of 1%. Clusters were defined on the basis of the 80% similarity cutoff.

Statistical analysis. Statistical analysis of the results relating to antimicrobial resistance of *V. parahaemolyticus* by sample type or country of origin was carried out using Fisher's exact test for 2 by 2 contingency tables (Statistica, Krakow, Poland). *P* values of <0.05 were considered significant.

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