Research Note

Prevalence and Antimicrobial Resistance of *Vibrio parahaemolyticus* Isolated from Raw Shellfish in Poland

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ABSTRACT

*Vibrio parahaemolyticus* is a marine bacterium recognized as an important cause of gastroenteritis in humans consuming contaminated shellfish. In recent years, increasing resistance to ampicillin and aminoglycosides has been observed among *V. parahaemolyticus* isolates. However, the first-line antimicrobials such as tetracyclines and fluoroquinolones remained highly effective against these bacteria. The aim of this study was to evaluate the occurrence of *V. parahaemolyticus* in live bivalve molluscs available on the Polish market and to determine the antimicrobial resistance of the recovered isolates. A total of 400 shellfish samples (mussels, oysters, clams, and scallops) from 2009 to 2012 were tested using the International Organization for Standardization standard 21872-1 method and PCR for the species-specific toxR gene. Antimicrobial susceptibility of the isolates was determined using a microbroth dilution method. *V. parahaemolyticus* was identified in 70 (17.5%) of the 400 samples, and the toxR gene was confirmed in 64 (91.4%) of these isolates. Most of the isolates were recovered from clams (31 isolates; 48.4% prevalence) followed by mussels (17 isolates; 26.6% prevalence). More *V. parahaemolyticus*–positive samples were found between May and September (22.7% prevalence) than between October and April (11.4% prevalence). Antibiotic profiling revealed that most isolates were resistant to ampicillin (56 isolates; 87.5%) and to streptomycin (45 isolates; 70.3%), but all of them were susceptible to tetracycline and chloramphenicol. Forty-one isolates (64.1%) were resistant to two or more antimicrobials; however, only one isolate (1.6%) was resistant to three antimicrobial classes. The antimicrobials used in treatment of human *V. parahaemolyticus* infection had high efficacy against the bacterial isolates tested. This study is the first concerning antibiotic resistance of *V. parahaemolyticus* isolates in Poland, and the results obtained indicate that these bacteria may pose a health risk to consumers.

*Vibrio parahaemolyticus* is a gram-negative halophilic bacterium that naturally occurs worldwide in marine and estuarine environments (8, 23). This microorganism is recognized as one of the most important causes of foodborne illness in Asia and North America (23, 29). Every year about 50 to 70% of gastroenteritis cases in Japan and other Asian countries are caused by *V. parahaemolyticus* (23). According to data published by Centers for Disease Control and Prevention (5), 944 *Vibrio* infections (excluding toxigenic *V. cholerae* O1 and O139) were reported in 2012 in the United States, and *V. parahaemolyticus* was the most frequently isolated bacterial species (from 431 patients; 45.7%). In recent years, a significant increase of food poisoning caused by *V. parahaemolyticus* was also observed in Europe (2). One of the reasons for this may be change in eating habits associated with consumption of fish and shellfish. Climate change leads to the appearance of these bacteria in seawaters in which, due to low temperatures, it did not usually occur (2, 16). Anomalies in seawater temperature also may explain the sudden emergence of *Vibrio* outbreaks in countries such as France, Italy, and Spain (15, 20, 25, 29). Clinical symptoms of *V. parahaemolyticus* infection are watery diarrhea, nausea, and abdominal cramps. In the acute form of the disease, hemorrhagic gastroenteritis with bloody diarrhea occurs. A complication such as septicemia also may appear and sometimes lead to death (15, 23). Although most *V. parahaemolyticus* infections are self-limiting and rehydration therapy is sufficient, in some cases the use of antibiotics is necessary. Tetracyclines have been recommended as the antimicrobials of choice for treatment of severe *Vibrio* infections, but third-generation cephalosporins with doxycycline or fluoroquinolone alone are sometimes used (10). *V. parahaemolyticus* isolates from seafood and the environment are commonly resistant to ampicillin, and increasing resistance of these bacteria to aminoglycosides (streptomycin and gentamicin), tetracyclines, ciprofloxacin, and chloramphenicol has been observed worldwide (1, 10, 13, 18). Although human clinical isolates have lower resistance to antibiotics than do the environmental strains, β-lactam–resistant *V. parahaemolyticus* isolates also have been reported (21, 28).

The aim of this study was to investigate the prevalence of *V. parahaemolyticus* in live bivalve molluscs available in Polish markets and to evaluate the antimicrobial resistance of these isolates.

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MATERIALS AND METHODS

Collection of samples. A total of 400 raw samples of bivalve molluscs were collected between 2009 and 2012: mussels (n = 128), oysters (n = 107), clams (n = 120), and scallops (n = 45) (Table 1). All shellfish were purchased in Polish markets and originated from various European countries: the Netherlands (n = 181), Norway (n = 99), Italy (n = 59), France (n = 50), and others (n = 11).

Bacteriological examinations. Microbiological analysis was performed according to the International Organization for Standardization (ISO) standard 21872-1 (11). A 25-g sample (pooled mussel flesh and intravalvular liquid) was homogenized in 225 ml of alkaline peptone water containing 1% NaCl (ASPW) and incubated at 41.5 ± 1°C for 6 ± 1 h (first enrichment). Then, 1 ml of this culture was transferred to 9 ml of ASPW and incubated at 41.5 ± 1°C for 18 ± 1 h (second enrichment). One loopful of the first and second enrichment broths were streaked on two selective media, thiosulfate citrate bile sucrose agar (Merck, Darmstadt, Germany) and chromogenic medium chromID Vibrio agar (bioMérieux, Marcy l’Étoile, France), and incubated at 37 ± 1°C for 24 ± 2 h. Suspected V. parahaemolyticus colonies were subcultured on saline nutrient agar with 1% NaCl (SNA) and confirmed by morphology, Gram staining, motility, and an oxidase test. All the presumptive V. parahaemolyticus isolates were then identified based on the API 32E system (bioMérieux) and a halotolerance test with various concentrations of NaCl (0, 2, 6, 8, and 10%).

Identification of V. parahaemolyticus by PCR. The seventy isolates recognized as V. parahaemolyticus by the biochemical tests were confirmed by a PCR method for the detection of the species-specific toxR gene (12). One bacterial colony was suspended in 1 ml of sterile water and centrifuged at 13,000 × g for 1 min. DNA was extracted using the GenomicMini kit (A&A Biotechnology, Gdynia, Poland) according to the producer’s instructions. The PCR was performed in a thermocycler (Biometra GmbH, Goettingen, Germany) as follows: 5 min of initial denaturation at 96°C; 30 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1.5 min, and extension at 72°C for 1.5 min; and a final extension at 72°C for 7 min. The PCR products were stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO) and visualized in 1.5% agarose gels (Sigma-Aldrich) using the Gel Doc 2000 documentation system (Bio-Rad, Hercules, CA). The confirmed V. parahaemolyticus isolates were stored at -80°C until further analysis.

Determination of antimicrobial resistance. Sixty-four V. parahaemolyticus isolates were subcultured twice on SNA and incubated at 37°C for 24 ± 2 h. Susceptibility tests were performed using a bacterial inoculum with a turbidity equivalent to a 0.5 McFarland standard. Fifty microliters of the suspension was transferred to 11 ml of Mueller-Hinton broth with 1% NaCl, and antimicrobial plates containing the following antimicrobials (Trek Diagnostic Systems, East Grinstead, UK) were inoculated: ampicillin, ciprofloxacin, gentamicin, tetracycline, streptomycin, and chloramphenicol. The antibiotics used in the study were chosen in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations (6), including antimicrobials used in treatment of Vibrio infections. Escherichia coli ATCC 25922 was used in each analysis as an antibiotic quality control. V. parahaemolyticus ATCC 17802 was used as the reference strain with the following MICs: ampicillin, >32 mg/liter; chloramphenicol, <2 mg/liter; ciprofloxacin, 0.25 mg/liter; gentamicin, 8 mg/liter; streptomycin, 64 mg/liter; and tetracycline, <1 mg/liter. After incubation at 37°C for 24 ± 2 h, the MICs were read with the Vision system (Trek), and the results were interpreted according to CLSI guidelines (6). The breakpoint for streptomycin, which was not established by the CLSI, was derived from earlier studies (1, 28).

Statistical analysis. Statistical analyses of the results relating to the seasonal prevalence of V. parahaemolyticus in shellfish and to antimicrobial resistance by shellfish type or country of origin were performed using a 2 × 2 contingency table and Fisher’s exact test (Statistica, Krakow, Poland). P values were two-tailed, and groups were considered significantly different at P < 0.05.

RESULTS AND DISCUSSION

Prevalence of V. parahaemolyticus. During the study period, a total of 400 shellfish samples were tested for the presence of V. parahaemolyticus. With the ISO method, these bacteria were identified in 70 (17.5%) of the 400 samples (Table 1). However, the PCR assay for the toxR gene confirmed only 64 (91.4%) of these isolates as V. parahaemolyticus. Of the four kinds of shellfish tested, clams were most commonly contaminated (48.4% of samples positive) followed by mussels (26.6%) and oysters (18.8%). In a similar study conducted between 2006 and 2008 by Roque et al. (26), V. parahaemolyticus was mainly isolated from oysters (65 [42.2%] of 154 total samples were positive) followed mussels (51 [33.1%]) and clams (38 [24.7%]). In the present study, most of the contaminated molluscs originated from the Netherlands (29 [45.3%] of 64 samples) and Italy (23 [35.9%]). Some shellfish from Norway (8 [12.5%]) and France (4 [6.3%]) also were positive for this pathogen. Similar results were obtained by other authors in Europe; V. parahaemolyticus was isolated from 10.3% of 885 mussel samples in Norway and in 12.5% of 1,551 mussel samples in Spain (3, 17). However, a higher prevalence of V. parahaemolyticus was observed in shellfish from France (27): 23.3% in mussels and 43.9% in oysters.
In contrast to the results from European countries, V. parahaemolyticus was more commonly detected in raw bivalve molluscs in Asia. In India, this pathogen was identified in 75.9% (63 of 83) of samples based on microbiological and PCR data for the toxR gene (24). Similar results were obtained by Yu et al. (32) in Taiwan, where V. parahaemolyticus were found in 70.8% of oyster samples and 68.8% of clam samples.

The prevalence of these bacteria in the environment appears to be correlated with geographical area, season, and type of shellfish tested. A significant increase in the occurrence of V. parahaemolyticus was observed in warmer months with water temperatures >15°C. In the present study, conducted in 2009 through 2012, a different number of samples was tested at two times of the year: warmer (May through September) and colder (October through April) months (Table 2). When the total results were compared, significant differences (P < 0.05) in the prevalence of these bacteria were found, i.e., V. parahaemolyticus was detected in 22.7 and 11.4% of samples tested, respectively. In a similar study performed with Adriatic mussels, Ottaviani et al. (22) isolated these microorganisms during the warmer months (May through September), at a comparable prevalence (24.3%) as identified in the present investigation. In a study in Iran (33), 11.0% of molluscs were positive for V. parahaemolyticus, and seasonal differences in the prevalence of these bacteria were observed, e.g., more shellfish were contaminated during spring and summer than during autumn and winter periods. All these findings support seasonal variation in the occurrence of V. parahaemolyticus.

**Antimicrobial resistance.** Antimicrobial resistance among the V. parahaemolyticus tested revealed that most isolates (87.5%) were resistant to ampicillin (56 of 64 isolates; Table 3). These data are similar to the results from other countries where V. parahaemolyticus of shellfish origin were mainly resistant to β-lactams (ampicillin) (9, 10, 13, 19, 31). In other investigations, especially from Asia, 100% of isolates were resistant to this antimicrobial (14, 30). However, Daramola et al. (7) reported low resistance (only 1.3%) to β-lactams in environmental V. parahaemolyticus isolates in England. Shaw et al. (28) found that more environmental V. parahaemolyticus isolates (51.9%, 40 of 77) were resistant to ampicillin compared with clinical isolates (25.0%). However, Ottaviani et al. (21) found that all strains from both shellfish and clinical samples were resistant to this antimicrobial. The relatively low percentage of isolates resistant to gentamicin (10.9%) identified in the present investigation is in contrast to the results of other studies performed in Asia and Europe. Liu et al. (14) found 34.2% of V. parahaemolyticus of seafood origin were resistant to gentamicin, whereas Daramola et al. (7) reported that 73.7% of tested isolates were resistant to gentamicin. A high level of resistance to streptomycin (70.3%) also was observed among the isolates examined. Li et al. (13) reported that 100% of Vibrio isolates were susceptible to this antibiotic. In similar studies conducted in Korea (18) and Italy (19), 8.7 and 85.7% of V. parahaemolyticus isolates, respectively, were resistant to aminoglycosides (streptomycin). The high resistance to β-lactams or aminoglycosides observed among V. parahaemolyticus may be associated with the extensive use of these antimicrobials in treatment of human infections and agriculture and aquaculture during recent decades (4, 10). In water environments, bacteria, including V. parahaemolyticus, from different sources were able to exchange their genetic resistance determinants, e.g., those located on plasmids, which may increase the antibiotic resistance of these microorganisms (13, 21). In the present investigation, only one isolate (1.6%) was resistant to fluoroquinolones (ciprofloxacin), whereas in similar studies from Hong Kong (14) and the United Kingdom (7) 26.3 and 9.2% of such isolates, respectively, were resistant to fluoroquinolones. However, the sources of the bacteria tested were not the same, making it difficult to directly compare these results. All V. parahaemolyticus isolates analyzed in the present investigation were sensitive to chloramphenicol.

<table>
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<tr>
<th>TABLE 2. Seasonal prevalence of V. parahaemolyticus</th>
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<tr>
<td><strong>No. of positive samples/no. tested (% positive)</strong></td>
</tr>
<tr>
<td><strong>Sampling year</strong></td>
</tr>
<tr>
<td>2009</td>
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<td>2010</td>
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<tr>
<td>2011</td>
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<td>2012</td>
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<td><strong>Total</strong></td>
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<th>TABLE 3. Antimicrobial resistance of V. parahaemolyticus strains tested</th>
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<tr>
<td><strong>Antimicrobial class</strong></td>
</tr>
<tr>
<td>β-Lactams</td>
</tr>
<tr>
<td>Chloramphenicols</td>
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<tr>
<td>Fluoroquinolones</td>
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<tr>
<td>Aminoglycosides</td>
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<td>Tetracyclines</td>
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* MICs and cutoff values for resistance were according to CLSI guideline M45-A2 (6); for streptomycin, these values were as described by Baker-Austin et al. (1) and Shaw et al. (28).
or to more than one class of antibiotics. These findings
Polish markets, especially during warmer months, and
V. parahaemolyticus
that
efficacy against
i.e., those used in the treatment of human infections (e.g.,
two or more agents, these isolates originated from the
cosides. Although most
V. parahaemolyticus
b
antimicrobials: fluoroquinolones,
resistance pattern, i.e., resistance to three classes of
V. parahaemolyticus
ampicillin and streptomycin (33 isolates; 51.6
resistance pattern observed was the combination of
resistant to two or more antimicrobials. The most common
isolates in relation to the sample type and country
molyticus
(19).
Analysis of the relationship between the type of
shellfish and antimicrobial resistance revealed that clams
were significantly more often contaminated ($P < 0.05$) with
V. parahaemolyticus
strains that were resistant to streptomycin (80.6% of isolates) than were oysters (41.7% of isolates). Significant differences ($P < 0.05$) in the prevalence of streptomycin-resistant isolates also were
found for Italian shellfish (87.0%) and shellfish from the
Netherlands (58.6%).

Antimicrobial resistance patterns among V. parahaemolyticus isolates in relation to the sample type and country of origin are presented in Table 4: 41 (64.1%) isolates were resistant to two or more antimicrobials. The most common resistance pattern observed was the combination of ampicillin and streptomycin (33 isolates; 51.6%). Only one V. parahaemolyticus isolate (1.6%) displayed a multi-resistance pattern, i.e., resistance to three classes of antimicrobials: fluoroquinolones, $\beta$-lactams, and aminoglycosides. Although most V. parahaemolyticus (78.9%) isolates evaluated by Daramola et al. (7) were resistant to two or more agents, these isolates originated from the environment. In the present study, the first-line antibiotics, i.e., those used in the treatment of human infections (e.g., tetracyclines, chloramphenicol, and ciprofloxacin) had high efficacy against V. parahaemolyticus isolated from shellfish.

In conclusion, the results of the present study indicated that V. parahaemolyticus is present in shellfish available in Polish markets, especially during warmer months, and a high percentage of the isolates were resistant to ampicillin or to more than one class of antibiotics. These findings suggest that the consumption of raw or poorly cooked shellfish may pose a risk for consumers.

ACKNOWLEDGMENT
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REFERENCES

| Table 4. Antimicrobial resistance patterns among 64 V. parahaemolyticus isolates tested |
|---------------------------------|---------------------------------|-----------------|
| Resistance pattern (no. of antimicrobial classes)$^a$ | Sample type$^b$ | Country of origin$^c$ | Total (%) |
| Sensitive to all | 2 (O), 1 (C) | 2 (NL), 1 (I) | 3 (4.7) |
| AMP (1) | 6 (M), 5 (O), 5 (C) | 10 (NL), 2 (I), 2 (N), 2 (F) | 16 (25.0) |
| STR (1) | 3 (M), 1 (C) | 3 (NL), 1 (I) | 4 (6.3) |
| STR, GEN (1) | 1 (C) | 1 (I) | 1 (1.6) |
| AMP, STR (2) | 18 (C), 7 (M), 4 (O), 4 (S) | 14 (I), 11 (NL), 6 (N), 2 (F) | 33 (51.6) |
| AMP, STR, GEN (2) | 4 (C), 1 (M), 1 (O) | 3 (I), 3 (NL) | 6 (9.4) |
| AMP, STR, CIP (3) | 1 (C) | 1 (I) | 1 (1.6) |

$^a$ Abbreviations for antimicrobials are given in Table 3.
$^b$ O, oysters; C, clams; M, mussels; S, scallops.
$^c$ NL, The Netherlands; I, Italy; N, Norway; F, France.

and tetracycline. In other studies, up to 100% of isolates were resistant to chloramphenicol (7), although the percentage of resistant V. parahaemolyticus usually ranged between 0% (7, 19) and 35.0% (31). Some authors (7, 31) found some isolates (from 20 to 53%) that were resistant to tetracyclines, whereas in other studies all isolates were susceptible to this antibiotic (19).

Analysis of the relationship between the type of shellfish and antimicrobial resistance revealed that clams were significantly more often contaminated ($P < 0.05$) with V. parahaemolyticus strains that were resistant to streptomycin (80.6% of isolates) than were oysters (41.7% of isolates). Significant differences ($P < 0.05$) in the prevalence of streptomycin-resistant isolates also were found for Italian shellfish (87.0%) and shellfish from the Netherlands (58.6%).

Antimicrobial resistance patterns among V. parahaemolyticus isolates in relation to the sample type and country of origin are presented in Table 4: 41 (64.1%) isolates were resistant to two or more antimicrobials. The most common resistance pattern observed was the combination of ampicillin and streptomycin (33 isolates; 51.6%). Only one V. parahaemolyticus isolate (1.6%) displayed a multi-resistance pattern, i.e., resistance to three classes of antimicrobials: fluoroquinolones, $\beta$-lactams, and aminoglycosides. Although most V. parahaemolyticus (78.9%) isolates evaluated by Daramola et al. (7) were resistant to two or more agents, these isolates originated from the environment. In the present study, the first-line antibiotics, i.e., those used in the treatment of human infections (e.g., tetracyclines, chloramphenicol, and ciprofloxacin) had high efficacy against V. parahaemolyticus isolated from shellfish.

In conclusion, the results of the present study indicated that V. parahaemolyticus is present in shellfish available in Polish markets, especially during warmer months, and a high percentage of the isolates were resistant to ampicillin or to more than one class of antibiotics. These findings suggest that the consumption of raw or poorly cooked shellfish may pose a risk for consumers.