Identification of tdh-positive Vibrio parahaemolyticus from an outbreak associated with raw oyster consumption in Spain

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Abstract

Between August and September 1999, a total of 64 cases of illness were identified in three episodes of acute gastroenteritis associated with the consumption of live oysters from a typical outdoor street market in Galicia (northwest Spain). Nine case patients were hospitalized and analysis of their stool samples revealed the presence of Vibrio parahaemolyticus. The strains isolated from two stool samples were studied for antibiotic susceptibility, biochemical characteristics and presence of virulence factors. Both isolates were Kanagawa phenomenon positive and produced thermostable direct hemolysin, which is related to pathogenicity in humans. These results show the presence of pathogenic V. parahaemolyticus in mollusks harvested in Europe and reveal the risk of illness associated with their consumption, suggesting the revision of V. parahaemolyticus risk assessment associated with consumption of raw live shellfish.

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Keywords: Shellfish; Oyster; Outbreak; Molluscan consumption; Risk assignment; Vibrio parahaemolyticus

1. Introduction

Vibrio parahaemolyticus is a halophilic organism that inhabits marine and estuarine environments. It was isolated for the first time in Japan in 1950 associated with an outbreak of food poisoning [1]. Foodborne infections by this organism cause gastroenteritis as the most common clinical presentation. Other symptoms include abdominal cramps, nausea, vomiting, headache, fever and bloody diarrhoea, while sepsis is quite rare [2]. V. parahaemolyticus has been recognized as an important cause of foodborne illness in Asia and United States and its outbreaks have been associated with consumption of raw or undercooked shellfish [3]. However, diseases caused by V. parahaemolyticus in Europe have rarely been reported. The most important reported outbreaks occurred in France and were related to the consumption of seafood imported from Asia [4]. In Spain, eight cases of acute gastroenteritis caused by V. parahaemolyticus in humans were reported in 1989 [5]. All cases were associated with fish or shellfish ingestion, and one case was specifically related with the consumption of live clams.

The pathogenesis of V. parahaemolyticus has been based on the presence of virulence factors, especially thermostable direct hemolysin (TDH) [6,7]. TDH causes beta-haemolysis of human erythrocytes in agar medium, a reaction known as the Kanagawa phenomenon (KP) [7]. The association between KP positivity of a strain and its ability to cause gastroenteritis has been well established [8] and the presence of the tdh gene is used routinely to determine the pathogenicity of V. parahaemolyticus strains [9].

The present study describes the first reported outbreak of V. parahaemolyticus associated with consumption of raw oysters in Europe, and includes additional data about antibiotic susceptibility, biochemical characteristics and presence of virulence factors in the outbreak strains.

2. Materials and methods

During summer 1999, the Galician Regional Department of Public Health received information from public hospitals on acute gastroenteritis. The illnesses were char-
characterized during diarrhea of brief duration. Stool samples were collected during the outbreak from patients with acute gastroenteritis and were examined to determine the etiological agent. All the cases were related with the ingestion of live mollusks harvested in Galicia (Spain) in a typical outdoor street market in Vigo (Galicia). The surveillance report only included data on demographics (age and sex), hospital and microorganism isolated from the stool.

For *V. parahaemolyticus* investigation, clinical stool specimens collected from patients during the illness outbreaks were added to 100 ml of alkaline peptone water and incubated at 37°C for 18 h. A loopful of the enrichment culture was streaked on thiosulfate-citrate-bile-sucrose (TCBS) agar, and incubated at 37°C for 18–24 h. The bacterial colonies were randomly selected and subjected to species identification by biochemical tests on API 20E strip (BioMérieux, Marcy-l’Etoile, France) and other complementary biochemical assays. The isolated strains were stored at −80°C for further analysis. For recovery, bacteria were cultured on tryptic soy agar containing 3% NaCl at 37°C for 24 h.

Antibiotic susceptibility was determined using the disk diffusion method. An antibiotic-loaded paper disk (Difco) was placed on a Mueller–Hinton agar plate with a bacterial lawn (three replicates). After incubation at 37°C for 18–24 h, the size of the inhibition zone was recorded and interpreted according to the reference provided by the manufacturer. The antibiotics evaluated were ampicillin, amoxicillin-clavulan, ceftriaxone, cefotaxime, cefoxitin, ceftazolin, ciprofloxacin, cefuroxime, erythromycin, gentamicin, streptomycin, tetracycline, trimethoprim/sulfamethoxazole and vancomycin.

*V. parahaemolyticus* is not included in the normal microbiological surveillance system for infectious gastroenteritis. Therefore, it is not investigated in routine in foodborne outbreaks and when it has been detected, the strains have not been usually stored. For this reason, after an intensive search in all the hospitals involved in the outbreak, only two isolates of *V. parahaemolyticus* were obtained. These two strains had been stored in the Meixoeiro Hospital, located in the city where the outbreak occurred. These strains were employed for biochemical characterization and study for the presence of virulence factors. Two additional *V. parahaemolyticus* cultures isolated from patients in Madrid and Barcelona in the same period were included in this study, as well as nine environmental strains isolated in our laboratory from marine samples (seawater and mollusks) during the monitoring of *V. parahaemolyticus* in the coastal waters of Galicia.

The KP was studied by culturing the strains on Wagatsuma agar containing 1–2% washed human type O erythrocytes at 37°C for 18–24 h. Colonies with a clear beta-hemolytic zone were designated as KP positive.

Presence of the *tl*, *tdh* and *trh* genes in *V. parahaemolyticus* cultures was determined by polymerase chain reaction (PCR) according to the procedure described by Bej et al. [10]. The *tl* gene is specific to *V. parahaemolyticus* and it was used to confirm the recovered strain, while *tdh* and *trh* genes encode the TDH and TRH hemolysins that are correlated with the pathogenicity of *V. parahaemolyticus*. For DNA extraction, the isolate was cultured on a tryptic soy agar plate containing 3% NaCl at 37°C overnight. Several well-grown colonies were chosen and resuspended in 300 μl sterile distilled water and heated to 100°C for 30 min to lyse the cells. The lysate was centrifuged, and the supernatant was stored at −20°C. PCR assays were performed using the oligonucleotide primer pairs for *tdh* (5′-GTA AAG GTC TCT GAC TTT TGG AC-3′ and 5′-TGG AAT AGA ACC TTC ATC TTC ACC-3′), *trh* (5′-TTG GCT ATC ATA TTT TCA GTA TCT-3′ and 5′-GAT AAC CAT ATG CCC ATT TCC G-3′) and *tl* (5′-AAG GAT TAT GCA GAA GCA CTG-3′ and 5′-GCT ACT TTC TAG CAT TTT CTC TGC-3′). Each 50 μl of PCR mixture contained 0.5 μg of purified genomic DNA, 1 μM of each oligonucleotide primer, 50 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 μM of each dNTP and 2.5 U of AmpliTaq DNA polymerase (Bioline). PCR reactions were carried out in a PTC200 thermocycler (MJ Research) with the following reaction conditions: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and primer extension at 72°C for 1 min. A final extension was performed at 75°C for 5 min.

### 3. Results

Between August and September 1999, an outbreak with three episodes of illness was detected in Galicia (northwest Spain). Sixty-four outbreak-associated patients were identified (Table 1). Nine of the case patients were hospitalized. The most common symptom was diarrhea of brief duration. Additional symptoms included abdominal cramps, nausea, headache, fever and vomiting. The median duration of illness was 3 days. The patients presented onset diarrhea within 12–24 h of eating raw oysters in a typical outdoor street market in Vigo (Galicia-Spain). The analysis of stool samples from patients revealed the presence of *V. parahaemolyticus* in all cases. The case patients’ median age was 43 years and 51.5% were female. All the patients resided in two cities near the outbreak site in the south of Galicia.

Biochemical analysis showed that the outbreak strains

<table>
<thead>
<tr>
<th>Date</th>
<th>No. of persons ill</th>
<th>No. of persons hospitalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-08-99</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>30-08-99</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>04-09-99</td>
<td>35</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 1 Data for the three episodes of oyster-associated gastroenteritis in Spain
were positive for lysine and ornithine decarboxylase, indole, cytochrome oxidase, salt tolerance (growth in 3, 6 and 8% NaCl), glucose and mannitol fermentation, and negative for arginine dihydrolase, citrate, gelatinase and arabinose fermentation (Table 2). These data were coincident with the biochemical characterization of environmental strains from the marine environment tested in our laboratory, while they contrast with the reference strain ATCC43996 (KP positive) employed as a control, which was positive for citrate, gelatinase and arabinose fermentation.

The antibiotic sensitivity test of the outbreak strains carried out in triplicate plates showed that all strains were resistant to ampicillin, erythromycin, streptomycin, and vancomycin. They presented intermediate susceptibility to gentamicin, cefazolin and cefuroxime, and sensitivity to amoxicillin-clavulan, ceftriaxone, cefotaxime, cefoxitin, ciprofloxacin and trimethoprim/sulfame.

The outbreak strains were confirmed by PCR for presence of \( \text{tl} \) gene, which is specific for \( V. \) parahaemolyticus (Fig. 1). The pathogenicity of the strains isolated was investigated by the study of \( \text{tdh} \) and \( \text{trh} \) genes, which encode two hemolysins directly associated with pathogenic strains. The strains possessed the \( \text{tdh} \) gene and were KP positive, showing a clear zone of hemolysis larger than 3 mm, while \( \text{trh} \) genes were not detected. All strains were urease negative, the presence of which was correlated with the presence of \( \text{tdh} \) and \( \text{trh} \) genes in clinical strains isolated from the USA and Asia [11]. The two additional \( V. \) parahaemolyticus cultures included in this study from patients in Madrid and Barcelona showed the same biochemical and genetic characteristics as the outbreak strains.

### 4. Discussion

To our knowledge, the present study represents the first reported shellfish-associated outbreak by \( V. \) parahaemolyticus in Europe, but it also provides the first direct evidence of the presence of pathogenic TDH-positive \( V. \) parahaemolyticus in the European marine environment. \( V. \) parahaemolyticus has been frequently found in coastal waters and seafood in Europe as revealed by different studies [12]. However, these investigations only detected or enumerated the total numbers of \( V. \) parahaemolyticus, without considering their virulence.

Beside this lack of quantitative data on the presence of pathogenic \( V. \) parahaemolyticus, a review of the available epidemiological information shows that no \( V. \) parahaemolyticus outbreak associated with the consumption of shellfish has been reported in Europe for the last 30 year [2]. Therefore, the risk of infections caused by \( V. \) parahaemolyticus in Europe has been considered very low [12], especially compared with data on infection incidence in the USA and Asia. Nevertheless, this consideration may underestimate the real risk of \( V. \) parahaemolyticus infec-

### Table 2

<table>
<thead>
<tr>
<th>Biochemical characteristics</th>
<th>Virulence genes</th>
<th>KP</th>
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<tbody>
<tr>
<td>ADH</td>
<td>LDC</td>
<td>ODC</td>
</tr>
<tr>
<td>---</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>Outbreak strains</td>
<td>+</td>
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</tr>
<tr>
<td>Additional strains*</td>
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<td>+</td>
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<tr>
<td>Environmental strains</td>
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<td>+</td>
</tr>
<tr>
<td>ATCC43996</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; CIT, citrate; GEL, gelatinase; MAN, mannitol; ARA, arabinose; URE, urease; V: 40–50% of strains are positives.

*Strains from two patients from Madrid and Barcelona.
tion due to deficiencies in the monitoring and investigation of foodborne illness. *V. parahaemolyticus* is not included in the European Network for Epidemiologic Surveillance and Control of Communicable Diseases and it is also excluded from the microbiological surveillance system for infectious gastroenteritis [12]. For this reason, this pathogen is not investigated routinely in every clinical case of foodborne illness, monitoring being restricted to specific hospitals located in coastal areas with a tradition of shellfish consumption during summer. When *V. parahaemolyticus* is detected, the investigation is normally restricted to identification of the etiological agent, without monitoring the source of infection, the food implicated or the number and virulence of the isolated strains. This lack of information limits the value of epidemiological studies on infectious gastroenteritis and the food source, which could estimate the real hazard of *V. parahaemolyticus* associated with shellfish consumption and a true risk assessment.

The biochemical characteristics of the outbreak strains reveal a clearly different pattern to those present by the reference strains (Table 2). Strains of *V. parahaemolyticus* from the outbreak, and from patients in Madrid and Barcelona, showed similar biochemical reactions to environmental strains isolated in our laboratory from marine environments near the outbreak place. Gelatinase, citrate utilization and arabinose fermentation are characteristic of *V. parahaemolyticus* [9] and they are present in the reference strain. However, they were not present in the other strains studied. This biochemical trait seems to be confined to the local strains investigated and further research is currently in progress to ascertain its significance.

The prevalence or presence of *V. parahaemolyticus* has been related to environmental conditions. Data from Asia, the USA and France show that the isolations of *V. parahaemolyticus* are concentrated between July and October [3,4] and there is a correlation between *V. parahaemolyticus* infections and warmer months of the year. This seasonal pattern of disease of *V. parahaemolyticus* has been widely reported and is coincident with the data presented in this study. The infections detected in this oyster-associated outbreak were concentrated in less than 15 days during August, when the beginning of the rainy season caused a decrease in salinity, and coincided with warm seawater temperatures. This relationship between the presence of this microorganism, warm temperatures and lower salinity, which has been reported previously [13], should be used in adjusting monitoring program for *V. parahaemolyticus*. Moreover, the high environmental temperatures typical of the summer months could influence the increase in contamination during the transport and display of shellfish in the market.

The detection of TDH-positive *V. parahaemolyticus* in oysters harvested in this region requires revision of the European microbiological regulations on molluskan shellfish to include the routine study of this microorganism in harvesting areas and markets. Furthermore, this finding should also promote an update of the microbial surveillance system for infectious gastroenteritis to produce more reliable data to conduct a *V. parahaemolyticus* risk assessments and protect public health.

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**References**


