Pulsed-Field Gel Electrophoresis and PCR Characterization of Environmental *Vibrio parahaemolyticus* Strains of Different Origins[▽]

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The present study used pulsed-field gel electrophoresis (PFGE) characterization to examine the intraspecies variability and genetic relationships among environmental isolates of *Vibrio parahaemolyticus* from different European countries. This is first study performed on environmental *V. parahaemolyticus* that included more than one European country.

Pulsed-field gel electrophoresis (PFGE) is a discriminative molecular typing technique, which was used to evaluate the genetic diversity among *Vibrio parahaemolyticus* strains and to identify the relatedness of environmental and food strains with reference strains of known human-pathogenic significance (8, 12, 14, 18, 23, 24). Studies focusing on environmental isolates are scarce and limited to specific geographic areas (8).

The present study was conducted to investigate the distribution of pathogenicity markers of *V. parahaemolyticus* (*tdh* or *trh* genes) in isolates of environmental origin collected in Europe. PFGE was used to examine the intraspecies variability, genetic relationships among the isolates, and relationship between pathogenic and nonpathogenic strains.

The study was performed on 96 *V. parahaemolyticus* isolates from Spain, Italy, Portugal, and the United Kingdom (Fig. 1) collected from food matrices, environmental samples, and clinical cases between 1994 and 2009, including two reference strains (508 [CCUG 43364] and 578 [CNRVC010089]). Strain identification and characterization were performed by PCR and colony hybridization.

Total DNA was extracted using the Wizard genomic DNA purification kit (Promega, Italy) by following the instructions of the manufacturer or a published protocol (15). A one-step PCR for the detection of the *toxR* gene was performed to confirm species identity (6), using strain ATCC 43996 (American Type Culture Collection) and molecular-grade water as positive and negative controls, respectively. Detection of the *tdh* and *trh* genes was performed on all *toxR*-positive strains, according to the work of Bej et al. (4) (*tdh* gene detection) and Tada et al. (21) (*trh* gene detection). Strains ATCC 43996 and ATCC 17802 were used as positive controls for *tdh* and *trh*, respectively, and molecular-grade water was used as a negative

control. Strain identification and pathogenicity characters were confirmed by means of colony hybridization (20). Briefly, digoxigenin-labeled probes were designed based on the sequences of the *toxR*, *tdh*, *trh1*, and *trh2* genes. Isolates were plated on Tryptone soy agar (Oxoid) with 2% sodium chloride (TSA-S) and incubated overnight at 37°C. Filter preparation from plates and colorimetric detection (nitroblue tetrazolium [NBT]–5-bromo-4-chloro-3-indolylphosphate [BCIP]) were performed by following a previously published protocol (17). Hybridization was performed overnight at 54°C in 6 M urea hybridization buffer, and specificity was ensured by two stringency washes at 65°C. All 96 strains were confirmed to be *V. parahaemolyticus* by PCR and colony hybridization. Five strains were positive for the *tdh* gene and 26 for the *trh* gene (Fig. 2); among these strains, the 24 isolates collected from food and the environment possessed the *trh2* variant and the 2 reference strains possessed *trh1*, as determined by colony hybridization.

PFGE was performed according to the PulseNet protocol with the following minor modifications (11, 25). Strains were cultivated overnight on TSA-S, and after preparation, plugs were placed in lysis buffer with proteinase K and incubated for 1 h at 50°C with constant shaking. Lysis was followed by two washes in pentadistilled water and five 10-min washes with 15 ml of Tris-EDTA (TE) buffer at 50°C. DNA digestion with the restriction endonuclease NotI (25 U/plug; Fermentas, Germany) was performed at 37°C overnight. *Salmonella enterica* serovar Braenderup strain H9812, digested overnight at 37°C with XbaI, was used as a molecular weight marker. Plugs were embedded into 1% agarose gel, and restriction fragments were resolved by PFGE on a contour-clamped homogeneous electric field (CHEF) II Mapper system (Bio-Rad Laboratories) in 0.5× Tris-borate-EDTA (TBE) with 300 μM thiourea and the following running conditions: 6 V/cm for 18 h at 14°C, with a 2- to 40-s switch time and pump speed of 0.7 l/min. Gels were stained in ethidium bromide (25 min), destained in distilled water (45 min), and photographed under UV light. Restriction patterns were analyzed using BioNumerics software v.4.0 (Applied Maths, Belgium). Clustering was performed using the unweighted pair group method using average linkages

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FIG. 1. Strain geographic origins. (The template for the map was obtained from http://d-maps.com/carte.php?num_car=6027&lang=en.)

(UPGMA) and the Dice correlation coefficient with a position tolerance of 1.5%. Clusters were defined on the basis of the 80% similarity cutoff (8).

Ninety-two of the 96 strains were typeable by PFGE, while four isolates were untypeable, indicating a high intraspecies genetic diversity for *V. parahaemolyticus*. This agrees with current knowledge of this species, with which 115 pulsotypes (PTs) previously were obtained from 535 isolates (26) and 104 PTs were obtained from 132 typeable isolates (8). Furthermore, the present results show a high genetic diversity even among the isolates obtained from a single region (Ebro Delta), with 44 strains distributed among 10 of the 11 clusters, a phenomenon probably explained by horizontal transfer of genetic material (7).

The 78 detected PTs presented from 11 to 26 bands, with a median of 16. The minimum genetic similarity among strains was 63.4% (Fig. 2), and 85 of them were distributed into 11 clusters, with 7 isolates not associated with any cluster. All clinical strains (associated with disease outbreaks) grouped in cluster V, confirming the results by Wagley et al. (24), who used the same clinical strains as references.

The finding of similar strains in distant geographic locations has been reported (8, 22). This is the case for clusters IV and V, with isolates coming from four and five different geographic origins, respectively, and including both toxigenic and nontoxigenic strains. In previous studies, using the present methodology, toxigenic strains of environmental origin gathered in the

same cluster (8, 22). This is not the case in the present study, where *trh*-positive strains appear in more than one cluster. Some Spanish strains were isolated in different, nonconsecutive years (clonal couples 889-910 and 896-913), demonstrating the stable presence of *V. parahaemolyticus* strains in certain habitats (13). Persistence in the environment could also be a feature of toxigenic strains (i.e., those containing the *trh* gene), as cluster III connects *trh*-positive Ebro Delta strains isolated over the course of 4 years, which are characterized by little genetic diversity. Pulsotypes of *trh*-positive isolates were also found to be stable over time in Norway (8) and Italy (14), and this is also a common feature for other vibrios, such as *V. cholerae* (10).

Previous studies (2) showed, on the basis of published data, a distinct geographic distribution of *trh*-positive and *tdh*-positive populations across the coast of Europe, with the *trh*-positive population dominating in Northern Europe (English Channel, Atlantic coast of France) (9, 16) and Norway (3) and *tdh*-positive strains reported in higher frequency from environmental sources in countries surrounding the Mediterranean Sea (1, 5). The present study, however, supports results of Roque et al. (19), highlighting the presence of *trh*-positive strains in food and environmental samples collected from different areas of the Mediterranean Sea (Adriatic and Tyrrhenian Seas, Ebro Delta) and showing that the genetic variant most frequently detected is *trh2*.

In conclusion, this is first study performed on environmental

FIG. 2. PFGE dendrogram generated by BioNumerics software, showing the relationship of fingerprints for 92 *V. parahaemolyticus* isolates. All the isolates were *toxR* positive. The four untypeable strains had the following sources, countries of origin, years of isolation, and genetic traits: isolate 11, fishery products, Italy, 2001, *tdh* negative, *trh1* negative, *trh2* negative; isolate 484, shellfish, Adriatic sea, 2002, *tdh* negative, *trh1* negative, *trh2* negative; isolate 893, shellfish (*Crassostrea gigas*), Ebro Delta (Spain), 2006, *tdh* negative, *trh1* negative, *trh2* negative; and isolate 908, shellfish (*Mytilus galloprovincialis*), Ebro Delta (Spain), 2008, *tdh* negative, *trh1* negative, *trh2* negative.

V. parahaemolyticus isolates from more than one European country, reconfirming that PFGE is a good tool for molecular characterization, showing the higher frequency of the *trh2* variant than of the *trh1* variant, and emphasizing the importance of appropriate molecular methods in public health and surveillance programs.

Nucleotide sequence accession numbers. The probes used for this study were deposited in the NCBI database under accession numbers 10616115 to 10616118.

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