

Nontoxicogenic *Vibrio parahaemolyticus* Strains Causing Acute Gastroenteritis

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We investigated the virulence properties of four *Vibrio parahaemolyticus* strains causing acute gastroenteritis following consumption of indigenous mussels in Italy. The isolated strains were cytotoxic and adhesive but, surprisingly, lacked *tdh*, *trh*, and type three secretion system 2 (T3SS2) genes. We emphasize that nontoxicogenic *V. parahaemolyticus* can induce acute gastroenteritis, highlighting the need for more investigation of the pathogenicity of this microorganism.

Vibrio parahaemolyticus infections cause acute, self-limiting gastroenteritis, typically characterized by diarrhea, abdominal cramps, nausea, vomiting, fever, and chills, lasting 1 to 3 days. The onset usually occurs within 24 h of eating contaminated food. Cases are most commonly reported during the warmer months and are often associated with eating raw or undercooked shellfish or other cooked foods that have been cross-contaminated with raw shellfish (22). Pathogenicity of this microorganism is attributed to the production of a thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH) (13). Recently, type three secretion system 2 (T3SS2) of *V. parahaemolyticus* has been investigated as an additional potential indicator of strain virulence (2, 8, 17). Together, the *tdh*, *trh*, and T3SS2 genes are widely considered the predominant indicators of strain virulence for this microorganism (8). This is substantiated by the prevalence of these genetic markers in clinical *V. parahaemolyticus* isolates, as opposed to their infrequent detection in food and environmental samples (8).

For this report, we characterized four *V. parahaemolyticus* strains lacking *tdh*, *trh*, and T3SS2 genes isolated as the sole pathogen from patients affected by acute gastroenteritis with indigenous mussels as the most probable source of infection. On 20 September 2010 in Torino (northern Italy), 4 people were hospitalized with acute gastroenteritis. The first case involved a healthy 47-year-old man (patient A), while a family group outbreak involved another three healthy individuals: a 55-year-old woman (patient B), a 59-year-old man (patient C), and a 58-year-old man (patient D). Epidemiological information reported that none of the patients had recently traveled to other countries and that on 19 September they had eaten home-cooked mussels, probably undercooked. Mussels involved in both the sporadic case and the outbreak came from the same Italian growing area (North Adriatic), but it was not possible to trace the specific lots. Patient A and patients B, C, and D developed diarrhea, fever, abdominal cramps, nausea, and vomiting about 13 h and about 18 h after mussel consumption, respectively. On admission, the patients were treated with intravenous hydration, and the symptoms ceased within 48 h without the need for antibiotic therapy.

Stool specimens of patients were cultured on MacConkey agar, Salmonella-Shigella agar, Columbia blood agar, Hektoen agar, 5% sheep blood agar supplemented with ampicillin (30 µg/ml)

(ASBA 30), Yersinia cefsulodin-irgasan-novobiocin (CIN) agar (all these media were from bioMérieux, Marcy l'Etoile, France), thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, Milan, Italy), and Campylobacter agar (Becton, Dickinson, Sparks, MD). Diarrheagenic *Escherichia coli* strains were sought by multiplex PCR (19) and Shiga toxin (Stx)-producing *E. coli* (STEC) also by an ImmunoCard STAT! enterohemorrhagic *E. coli* (EHEC) assay (Meridian Biosciences, Cincinnati, OH). An enzyme immunoassay (Ridascreen; R-Biopharm, Germany) was used to detect *Clostridium perfringens* enterotoxin. Group A human rotavirus and enteric adenovirus types 40 and 41 were tested using an immunochromatographic technique (VIKIA Rota-Adeno; bioMérieux), while norovirus GI-GII and astrovirus were tested using a multiplex reverse transcription-PCR (RT-PCR) assay (Seeplex Diarrhea ACE Detection Panel V; Seegene, South Korea). A RT-PCR protocol based on the specific JV33-SR80 primer pair was performed to detect sapovirus (20). Finally, enteric parasites were sought by conventional microscopy and staining methods and by the use of an ImmunoCard STAT! *Crypto/Giardia* assay (Meridian Biosciences).

From feces of each patient, in the absence of other enteric pathogens, Gram-negative oxidase-positive sucrose-negative microorganisms were isolated on TCBS agar. Ten suspect colonies, randomly selected, of each patient's TCBS culture were subcultured on Trypticase soy agar (Oxoid) with 1% NaCl and each of them presumptively identified using an API ID 32 GN commercial system (bioMérieux) and a standardized biochemical protocol (10, 14). Moreover, PCR was used to determine, in all the colonies, the presence of *V. parahaemolyticus* species-specific genes (*toxR* and *tlh*) and to detect the genes encoding the thermostable direct hemolysin (*tdh*), the TDH-related hemolysin (*trh*), and T3SS2-specific genes T3SS2 α and T3SS2 β (11, 12, 15). A group-specific

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TABLE 1 Properties of *Vibrio parahaemolyticus* clinical isolates analyzed in this study^a

Strain	Serotype	Source	Origin	<i>toxR</i>	<i>tdh</i>	<i>trh</i>	T3SS2 α	T3SS2 β	Inv	Adh	Cyt	Rep-PCR pattern
3	O6:K46	Stool	Patient A	+	—	—	—	—	—	+	+	P1
8	OUT:KUT	Stool	Patient B	+	—	—	—	—	—	+	+	P2
23	OUT:KUT	Stool	Patient C	+	—	—	—	—	—	+	+	P2
24	OUT:KUT	Stool	Patient D	+	—	—	—	—	—	+	+	P2

^a +, presence; —, absence; Inv, invasiveness; Adh, adhesiveness; Cyt, cytotoxicity.

(GS)-PCR method to detect the pandemic marker ToxRS (15) and serotyping by a slide agglutination test (Denka; Seike Corp., Tokyo, Japan) were also performed (15). The following American Type Culture Collection (ATCC) *V. parahaemolyticus* strains were used as positive controls: strain ATCC BAA-238 for the *tdh*, T3SS2 α , and *toxRS* genes and strain ATCC 17802 for the *trh* and T3SS2 β genes. For all the isolates, as additional markers of virulence, analyses of cytotoxicity for Caco-2 cells, adhesiveness on HEp-2 cells, and invasiveness on HT29 cells were performed (5, 16). Repetitive-sequence-based PCR (rep-PCR) was used as method of molecular subtyping (DiversiLab; bioMérieux). Susceptibilities to 15 antimicrobial agents were examined using the disk diffusion method on Mueller-Hinton agar plates in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (4). Antibiotics tested were amoxicillin-clavulanic acid, ampicillin, cephalothin, cephalixin, cefoperazone, ciprofloxacin, colistin, chloramphenicol, doxycycline, erythromycin, gentamicin, kanamycin, meropenem, nitrofurantoin, tetracycline, and trimethoprim-sulfamethoxazole. Interpretative criteria for each antibiotic tested were as published in CLSI guidelines (4) or followed the recommendations of the antimicrobial agent suppliers.

Our results are summarized in Table 1. All 10 of the patient A colonies were identified by the biochemical protocol as *V. parahaemolyticus*. The species identification was confirmed by the presence of *toxR* and *tdh* genes. These isolates were serotyped as O6:K46 and were also characterized as cytotoxic, adhesive, *tdh* negative, *trh* negative, GS-PCR negative, T3SS2 α negative, T3SS2 β negative, and not invasive. Ten colonies from each positive culture of patients B, C, and D were identified by the biochemical protocol as *V. parahaemolyticus* and were serotyped as OUT-(untypeable):KUT(untypeable). The species identification was confirmed by the presence of *toxR* and *tdh* genes. These isolates were also characterized as cytotoxic, adhesive, *tdh* negative, *trh* negative, GS-PCR negative, T3SS2 α negative, T3SS2 β negative, and not invasive. rep-PCR patterns were identical for all the colonies of patient A (pattern P1). A unique rep-PCR pattern (pattern P2) was obtained for all the colonies of patients B, C, and D but was different from that of patient A. All the isolates of patient A were resistant to ampicillin, amoxicillin-clavulanic acid, cephalothin, cephalixin, erythromycin, and trimethoprim-sulfamethoxazole. All the isolates of patients B, C, and D were resistant to ampicillin, amoxicillin-clavulanic acid, cephalothin, cephalixin, ciprofloxacin, and erythromycin. In both the family group outbreak and the sporadic case, the incubation period and specific symptoms, as fever, allowed us to exclude the involvement of algal biotoxins and other chemical molecules.

V. parahaemolyticus was the unique pathogen isolated from all the specimens, but the isolates were, surprisingly, negative for *tdh*, *trh*, and T3SS2; therefore, according to previous studies, those are to be considered nonpathogenic. However, in agreement with our results, recent studies in which the presence of toxin and T3SS2

genes in clinical isolates has been examined have revealed an unexplained number of strains that lack these genes, suggesting that *V. parahaemolyticus* may harbor other virulence factors (1, 6, 7, 8, 18, 21). Again, it was also recently hypothesized that the isolation of strains lacking the virulence genes from individuals exhibiting symptoms of *V. parahaemolyticus* illness was most likely due to the simultaneous consumption and proliferation of virulent and avirulent strains in a human host (1). Consequently, analysis of a single isolate per patient may lead to misdiagnosis of the etiological agent due to sampling error if the infecting microbial population is not homogeneous (1). In our case, the homogeneous results of the biochemical and molecular characterization of 10 colonies for each stool sample allowed us to minimize the possibility of the presence of a heterogeneous population of *V. parahaemolyticus* in the feces of each patient. Also, we found the same isolate (identical rep-PCR patterns) in three different patients and it is unlikely that it corresponds to a nonvirulent strain. Moreover, all our isolates, although nontoxicogenic, showed a virulence potential, being adhesive and cytotoxic. This last evidence is in agreement with a recent review of the pathogenicity effects of individual factors in *V. parahaemolyticus* stating that the deletion of *tdh* and/or *trh* does not affect cytotoxicity and that other factors are involved in the virulence as well as the TDH and TRH toxins (2).

To our knowledge, this report documents the first clinical isolation of nontoxicogenic *V. parahaemolyticus* strains causing gastroenteritis in Italy and underlines that clinical isolates lacking *tdh*, *trh* and T3SS genes could have unknown virulence mechanisms responsible for diarrhea, even if we did not rule out the possibility that *in vivo* or *in vitro* changes (*tdh* or *trh* deletion) might have also occurred.

With the exception of pandemic clones (9), clinical isolates of *V. parahaemolyticus* usually show high variability of serotypes (8). However, recently, gastroenteritis due to nontoxicogenic strains serotyped as OUT:KUT has been reported (6, 21). With regard to this, we underline that our nontoxicogenic strain of *V. parahaemolyticus* involved in the family group outbreak was also serotyped as OUT:KUT. It may be interesting to compare the molecular profiles of these strains to assess the degree of genetic similarity. Alarmingly, the incidence of *V. parahaemolyticus* infections due to eating raw or undercooked shellfish has shown a sustained increase since 2000, as reported recently by FoodNet from the Centers for Disease Control and Prevention (CDC) (3), and to date, for risk analysis, only the *tdh* and *trh* genes have been used to estimate the load of pathogenic strains in shellfish.

In conclusion, we believe that this article will increase the scientific community awareness that the virulence potential and the effective epidemiological importance of nontoxicogenic *V. parahaemolyticus* strains must be better investigated to safeguard the health of consumers.

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