Phenotypic and Genotypic Characterization of *Salmonella enterica* Serotype Paratyphi B Isolates from Environmental and Human Sources in Galicia, Spain

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ABSTRACT

*Salmonella* serotype Paratyphi B isolates obtained from shellfish and human infections in Galicia (northwest Spain) from 1998 were investigated by different phenotypic and genetic methods to evaluate their systemic or enteric nature. Isolates were investigated for D-tartrate fermentation, presence of genes encoding the effector proteins sopE1 and avrA, pulsed-field gel electrophoresis profile, and antimicrobial susceptibility. Systemic variant strains (dT−) were the dominant among the marine environment isolates. All dT− isolates were sopE1 positive and avrA negative, presented an indistinguishable electrophoresis profile, and were grouped in a single cluster. More electrophoresis heterogeneity was observed among dT+ isolates. Only two isolates showed resistance to any of the 16 antibiotics included in our panel. The present study identified the marine environment as a potential natural source of systemic variant isolates of *Salmonella* Paratyphi B. The presence of systemic variant isolates of *Salmonella* Paratyphi B in the marine environment is of notable public health significance as a result of the potential risk of acquiring enteric fever linked to the consumption of raw shellfish.

*Salmonella* is a major cause of foodborne illness in humans and is a common organism present in the enteric flora of most animals (5). There are currently 2,541 different serotypes of *Salmonella* included in the Kaufmann-White scheme (22). Most of these serotypes can produce gastroenteritis in animals and humans. However, only a few serotypes are able to invade and multiply within the host tissues to cause enteric fever or septicemia, which has serious consequences for human health. Although the incidence of typhoid fever is decreasing globally, it remains a major public health problem in the developing world (21). *S. enterica* serotype Typhi is the causal agent of typhoid fever, although a very similar but often less severe typhoid-like enteric fever syndrome is caused by *S. enterica* serotypes Paratyphi A, Paratyphi C, and Sendai. Additionally, *S. enterica* serotype Paratyphi B is able to cause both enteric fever and gastroenteritis.

*Salmonella* Paratyphi B was first isolated from cases of typhoid-like enteric fever in 1896 and subsequently has been identified in association with less severe gastrointestinal disease (26). Although all strains of this serotype share identical somatic and flagellar antigens, isolates from enteric fever and gastroenteritis cases are normally discriminated by their capacity to ferment the dextrorotatory [L(+)]-tartrate (d-tartrate). On the basis of this biochemical trait, the d-tartrate-fermenting strains (dT+) were designated as *S. enterica* serotype Java (12), and those failing to use d-tartrate (dT−) were designated *S. enterica* serotype Paratyphi B sensu stricto (3, 24). Evaluation of different phenotypic methods to test d-tartrate fermentation has shown poor correlation and reproducibility (13). Furthermore, limitations concerning the interpretation of results from the d-tartrate fermentation tests have often been reported (3, 24). These deficiencies have promoted the development of more reliable molecular assays to discriminate among fermentative and nonfermentative isolates (13).

In addition to the epidemiological and biochemical heterogeneity, *Salmonella* Paratyphi B isolates also show a high level of variability in the presence and polymorphism of several virulence determinants (4, 9, 26), uncommon in other serotypes (18, 23). Molecular tests based on the differential characteristics of virulence genes present in isolates from systemic or enteric infections have been proposed recently to discriminate among *Salmonella* Paratyphi B variants (24). The presence of genes sopE1 and avrA, encoding two effector proteins of the SPI1-associated type III secretion system, has been suggested as a diagnostic tool for identifying the systemic or enteric nature of the *Salmonella* Paratyphi B isolates. The sopE1 gene is present and the avrA gene is absent in isolates from systemic infections, whereas the sopE1 gene is absent and the avrA gene is present among enteric variants.

*Salmonella* Paratyphi B dT− isolates are mainly recovered from humans and are only rarely isolated from animals and the environment; however, dT+ variants can be detected

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from all three sources (8). An increase in the number of reports of multidrug-resistant Paratyphi B dT+ in the Netherlands (28), Scotland (6), Germany (17), and Canada (19) has been observed. Eighty-eight percent of the Paratyphi B dT+ isolates investigated in Germany since 1994 presented a multiple-antibiotic resistance phenotype (17). Very little information exists on the presence and characteristics of the isolates belonging to this serotype in Spain. The aim of this study was to characterize Salmonella Paratyphi B isolates obtained from the marine environment and human infections in Galicia (northwest Spain) by different phenotypic and genetic methods. Additionally, the genetic diversity and clonal relationships among isolates from the different sources were also investigated.

**MATERIALS AND METHODS**

**Bacterial isolates.** A total of 16 Salmonella Paratyphi B isolates were included in the study (Table 1). Four of these represent all the Salmonella Paratyphi B isolates originated from the analysis of 6,317 samples of mollusks and seawater taken from 1998 to 2002 during previous studies (15, 16). The samples were collected in the mollusk production areas located in the four most important rias (estuaries similar to small fjords, which extend from east to west) of Galicia in northwestern Spain. Additionally, eight isolates from human infections (one from blood and seven from stool) were investigated. These isolates represented all the serotype Paratyphi B isolates obtained from human infections in the same region and submitted to the National Reference Laboratory for Salmonella (Centro Nacional de Microbiología, Madrid, Spain) for serotyping between 1998 and 2004. Finally, the reference strains NCTC03176 (dT+) and NCTC05706 (dT+) from the National Collection of Type Cultures (HPA Colindale, London, UK) and two isolates from Germany (dT+ and dT−) were also included in the study.

**Investigation of d-tartrate fermentation.** The use of d-tartrate was investigated by lead acetate test following the protocol described by Alfredsson et al. (2) with minor modifications. Culture medium was elaborated as described in previous studies (2, 13). The final broth, which had a characteristic blue color, was dispensed in 8-ml volumes in test tubes with cotton-wool stoppers. The cultures were incubated at 37°C for 3 and 6 days aerobically in static conditions. After the incubation period, the cultures were tested for d-tartrate utilization by the addition of a saturated aqueous lead acetate solution in the proportion of 0.1 ml/1 ml of culture. Change in the color of the media and precipitate formation served as criteria for discrimination among dT− and dT+ isolates. dT− isolate cultures showed a typical yellow color and a small white precipitate on the bottom of the tubes after addition of lead acetate, whereas dT+ cultures presented an unchanged color and a flabby white precipitate that extended along 80% of the culture.

**DNA isolation and PCR.** Chromosomal DNA isolation was performed from an overnight culture of Salmonella isolates in Luria-Bertani medium incubated at 37°C with moderate shaking. A 1-ml aliquot of the culture was transferred to a tube and centrifuged at 10,000 × g at 4°C for 5 min. The supernatant was carefully discarded, and the pellet was suspended in 300 μl of Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA [pH 8.0]). The tubes were incubated in boiling water for 10 min and centrifuged for 5 min at 14,000 × g at 4°C. Supernatants were carefully transferred to a new tube, and a 5-μl aliquot was used as the template for PCR amplifications.

A multiplex PCR for the discrimination of dT− and dT+ Paratyphi B isolates was performed according to the procedure previously described by Malorny et al. (13). The multiplex PCR included the primers 166/167 (13) specifically designed to discriminate d-tartrate-fermenting isolates, and the primers ST11/ST15 (1) to detect a Salmonella-specific DNA sequence. The primer set 166/167 produces an amplicon of 290 bp when the ATG start codon is present in the STM 3356 gene (dT−), and no PCR product when this start codon is absent (dT+). The second primer set (ST11/ST15) amplifies a 429-bp PCR fragment specifically in all Salmonella isolates and serves as an internal positive control.

Two additional PCR procedures to detect the genes encoding the effector proteins sopE1 and avrA were carried out as described elsewhere (23). The presence of the sopE1 gene was investigated with the primer set sopE-P4/sopE-M2, which amplified a 398-bp PCR product. The avrA gene was investigated with the primer set avrA-P4/avrA-M1, which generated an amplicon of 385 bp. PCR reactions were carried out with a PT-200 DNA Engine Thermal Cycler (MJ Research, Waltham, Mass.), and amplicons were analyzed in a 1.8% agarose gel.

**PFGE.** Pulsed-field gel electrophoresis (PFGE) was performed according to protocols described elsewhere (7) following a method described previously (14). Chromosomal DNA was digested with 50 U of XbaI (Promega, Southampton, UK). PFGE was performed on a CHEF DR III system (Bio-Rad, Hercules, Calif.) in 0.5× Tris-borate-EDTA (TBE) extended-range buffer (Bio-Rad) with recirculation at 14°C. DNA macrorestriction fragments were resolved on 1% SeaKem Gold Agarose (Cambrex, Rockland, Me.) in 0.5× TBE buffer. DNA from Salmonella Braenderup H9812 restricted with XbaI was used as a size marker. Pulse times were ramped from 2.2 to 63.8 s during an 18-h run at 60 V/cm. Macrogen restriction patterns were compared with the use of BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Different profiles were designated with the letters X (XbaI types) in accordance with the restriction patterns.

**Antimicrobial susceptibility testing.** The isolates were screened for susceptibility to a panel of 16 antibiotics on Mueller-Hinton agar (Oxoid, Basingstoke, Hampshire, UK) by a disk diffusion methodology as described by NCCLS guidelines (20). The following disks (Oxoid) were used: amikacin (30 μg), amoxicillin–clavulanic acid (30 μg), ampicillin (10 μg), apramycin (15 μg), chloramphenicol (10 μg), cephoterazone (30 μg), ceftazidime (30 μg), colistin (25 μg), furazolidone (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), neomycin (10 μg), streptomycin (25 μg), sulfamethoxazole-trimethoprim (25 μg), compound sulfonamide (300 μg), and tetracycline (10 μg).

**RESULTS**

Results obtained from the investigation of d-tartrate utilization by lead acetate test of the four S. enterica serotype Paratyphi B isolates from the marine environment showed that the three isolates obtained from mussels and oysters between 1999 and 2001 were d-tartrate negative, whereas another isolate recovered from mussels in 2002 was d-tartrate positive (Table 1). These four isolates were detected predominantly during the fall months in the same central area of the ria of Arousa. The human isolates 663/00 and 667/00, identified from blood and stool samples, respectively, were d-tartrate negative. Both isolates were isolated during the year 2000 in the city of Ourense. The rest of the human isolates were d-tartrate positive. The ref-
TABLE 1. Sources and characteristics of 16 isolates investigated in this studya

<table>
<thead>
<tr>
<th>Source</th>
<th>Isolate</th>
<th>Year of isolation</th>
<th>Origin</th>
<th>d-Tartrate Lead acetate test</th>
<th>PCR</th>
<th>Virulence genes</th>
<th>PFGE profile</th>
<th>Antibiotic resistance</th>
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<td>2002</td>
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<td>+</td>
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<td>–</td>
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<td>+</td>
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<td>+</td>
<td>–</td>
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<tr>
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<td>A Coruña</td>
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<td>–</td>
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<td>Pontevedra</td>
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<td>–</td>
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<td>–</td>
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<tr>
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<td>+</td>
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<td>S</td>
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<td>2000</td>
<td>Germany</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>X07</td>
<td>F</td>
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a +, positive; –, negative; S, streptomycin; NK, not known; F, furazolidone. Antibiotics assessed were ampicillin, amikacin, apramycin, amoxicillin-clavulanic acid, chloramphenicol, cefoperazone, cefadroxil, colistin, furazolidone, gentamicin, nalidixic acid, neomycin, streptomycin, sulfamethoxazole-trimethoprim, compound sulfonamides, and tetracycline.

ference strains and the isolates from Germany showed the expected characteristics. All the positive cultures were unequivocally identified by the yellow color and the production of a compact precipitate in the bottom of the tubes; negative cultures maintained their original blue color and formed a fluffy precipitate along the culture tube. No differences between the results obtained after 3 and 6 days of incubation were observed. Results from PCR assay to discriminate between D-tartrate and dT- isolates were in complete agreement with the results achieved by lead acetate test (Table 1). PCR amplification was confirmed by the generation of the Salmonella-specific amplicon included in the PCR multiplex assay as internal positive control.

The presence of the virulence genes sopE1 and avrA assessed by PCR is summarized in Table 1. All the D-tartrate-negative isolates produced the expected 398-bp amplicon with the primer set sopE-P4 and sopE-M2 and were negative for avrA. By contrast, D-tartrate-positive isolates were negative for the sopE1 gene, although they showed heterogeneous results when the avrA gene was investigated: five D-tartrate isolates (UCM-279, 1945/01, 4343/03, 880/04, and 98/1967) were positive for this gene, and the other four D-tartrate isolates (6046/01, 4701/03, 3200/04, and NCTC05706) were negative.

PFGE analysis allowed discrimination of seven different profiles (Table 1 and Fig. 1). All the D-tartrate-negative isolates shared an identical restriction pattern and were grouped in a unique cluster (X01). This X01 profile included the three D-tartrate isolates obtained from the marine environment, two human isolates detected in 2000, the German human strain 64/1354, and the reference strain NCTC03176. Analysis of the D-tartrate-positive isolates revealed some heterogeneity among restriction patterns. The X02 profile was the predominant among D-tartrate isolates. This profile was exclusively identified in four isolates (UCM-279, 1945/01, 4343/03, and 880/04) obtained in Galicia from mussels and humans. The other three D-tartrate isolates from Galicia were grouped in a cluster (similarity >80%), clearly separated from the German D-tartrate isolates and the reference strain NCTC05706.

Results from the antimicrobial susceptibility testing of all isolates are listed in Table 1. A total of 13 isolates were sensitive to all the 16 antibiotics included in the panel. The isolates 4701/03 and 64/1354 showed resistance to streptomycin, whereas isolate 98/1967 was resistant to furazolidone.

DISCUSSION

The phenotypic and genotypic analysis of the isolates included in this study has revealed that the D-tartrate systemic variant is the dominant among the Salmonella Paratyphi B isolates from the marine environment in the study region. These isolates were detected during three consecutive years, mainly during the autumn months, in a reduced area located in the center of the ría of Arousa, where shellfish are cultured on ropes hanging from floating platforms. This seasonal pattern coincides with the results of previous studies in the region that linked the presence of Salmonella in the
FIGURE 1. Representative Xba I PFGE patterns and clustering analysis of S. enterica serotype Paratyphi B isolates. D-Tartrate fermentation, origin, isolation years, PFGE type designations, and isolate source are indicated in right. Dendrogram was generated by BioNumerics software. Numbers at dendrogram roots show similarity value (similarity percentage) among identified clusters. Numerals at top indicate molecular weights (kbp). R strain, reference strain; NK, not known.

marine environment to heavy rains and winds during the fall months (15). The identification of systemic variant isolates from shellfish located in zones far away from the coastal line revealed the presence of important contamination events of this specific pathogen in the area, as well as a marked capacity for this organism to survive in saline environments. Microbiological contamination studies of seawater offer only a snapshot of the contamination events. The identification of *Salmonella* Paratyphi B in the coastal waters during occasional sampling over a long period could be preliminary evidence that contamination with this serotype is more common than previously thought. The presence of this virulent human pathogen in marine environments is of notable public health concern because of the potential risk of transmission of enteric fevers by the consumption of raw shellfish.

This dominance of dT among the marine environment isolates contrasts with the results obtained from the analysis of human isolates, with only two isolates detected in 2000 at Ourense city identified as dT. This city is located inland 200 mi (100 km) away from the coast. There is a lack of information about the epidemiology of *Salmonella* Paratyphi B in Spain. According to recent data from the Spanish Boletín Epidemiológico Semanal (Ministerio de Sanidad y Consumo, Madrid, Spain), the presence of *Salmonella* Paratyphi B is rarely reported among human and nonhuman *Salmonella* isolates, with only 9 of a total of 7,529 human isolates in 2001 and 2 environmental isolates in 2002, both from aquatic sources. Information on the natural reservoirs of *Salmonella* Paratyphi B is scarce, especially regarding the survival capabilities and routes of transmission of the D-tartrate-negative variants. Enteric *Salmonella* Paratyphi B (*S. enterica* serotype Java) is characterized by a ubiquitous nature. Isolates of this biotype have been detected in human and animal infections, as well as from food and water sources. However, little epidemiological information on D-tartrate-negative isolates exists. This is in part because most of the investigations are restricted to serotype level, without additional information on their pathogenicity potential. Food contaminated by asymptomatic carriers has been proposed as the major source of contamination by the systemic variant (10).

It is known that natural populations of *Salmonella* Paratyphi B are genotypically and phenotypically heterogeneous. On the basis of this observation, Kauffmann (11) and Barker et al. (3) suggested that *Salmonella* Paratyphi B comprises several groups with different evolutionary histories that have converged in a unique antigenic structure. Further investigations have suggested that the variability within this serotype could be related to the host specificity. Systemic infections are mostly confined to humans, whereas enteric infections occur in a wide range of animals as well as in humans. The variety of selection pressures due to the adaptation to different hosts could account for the genetic diversity among dT isolates (9).

To clarify these hypotheses, several studies on the genetic population structure of *Salmonella* Paratyphi B have been carried out. Most of the D-tartrate-negative strains have been identified by multilocus enzyme electrophoresis as members of the unique globally distribute clone electrophoretic type Pb 1 (27). By contrast, D-tartrate-positive strains are highly heterogeneous and they are grouped in different clusters, although a study of isolates from France and the United States showed that 84% of the isolates be-
longed to only two predominant clusters (27). These results were additionally confirmed by the use of restriction fragment length polymorphisms, 16S ribosomal RNA genes, IS200 (9), fluorescent amplified fragment length polymorphism (25), and PFGE (10). The results obtained from PFGE analysis in the present study have shown a similar pattern of genetic relationships. All the D-tartrate-negative isolates presented an indistinguishable PFGE profile and were grouped in a unique cluster. This group included isolates obtained of local origin as well as others from Germany and the United Kingdom, which suggests that all these isolates could belong to the globally distributed clone. More heterogeneity was observed among D-tartrate-positive isolates from Galicia. D+ Spanish isolates were included in two unrelated groups clearly separated from isolates from Germany and the United Kingdom. Three human isolates from A Coruña city showed PFGE patterns indistinguishable from the UCM-279 mussel isolate. The presence of indistinguishable PFGE fingerprints in D+ isolates from human and mollusk origin could reveal preliminary evidence of a foodborne infection by the enteric variant of this serotype as a result of shellfish consumption.

Data from several previous studies have shown the emergence of multidrug-resistant clones among S. enterica serotype Paratyphi B D+ in an important number of European countries and Canada during the last decade (6, 17, 19, 28). Data of antibiotic resistance of the Paratyphi B isolates in this study revealed that most of the isolates from the environment and human sources are sensitive to the range of antibiotic tested, and only three isolates presented a very limited resistance. These results suggest that the dissemination of these multiresistant clones of this serotype has not reached this geographical region of Spain.

The present study has showed the suitability of PCR assays for a rapid and reliable evaluation of the systemic or enteric nature of the Salmonella Paratyphi B isolates. The results obtained from isolate characterization have identified the marine environment as a potential natural source of systemic variant isolates of Salmonella Paratyphi B. This finding is of notable public health importance because it demonstrates the potential risk of acquiring enteric fever linked to the consumption of live molluscan shellfish.

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REFERENCES


