Comparative Analysis of Shigella boydii 18 Foodborne Outbreak Isolate and Related Enteric Bacteria: Role of rpoS and adiA in Acid Stress Response

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ABSTRACT

Shigella boydii CDPH (Chicago Department of Public Health) serotype 18 was implicated in an outbreak of foodborne illness in 1998. The suspected food vehicles were parsley and cilantro imported from Mexico used to prepare bean salad. Previous studies revealed that S. boydii CDPH serotype 18 can survive in bean salad, which contains organic acids and whose pH decreases over time. Acid challenge assays in acidified tryptic soy broth at pH 4.5, acidified Luria-Bertani broth at pH 4.5, and acidified M9 minimal salts medium at pH 2.5 containing amino acids, arginine, or glutamic acid were performed using S. boydii CDPH, S. boydii ATCC 35966, S. flexneri 3136, Escherichia coli O157:H7 dd8872, and E. coli O157:H7 dd642 to compare differences in acid tolerance. Differences in survival of exponential-phase cells were detected in acidified tryptic soy broth and Luria-Bertani broth at pH 4.5. In acidified minimal medium containing arginine, S. boydii strains were able to survive at pH 2.5. The arginine decarboxylase gene (adiA) present in S. boydii is involved in survival at extremely low pH. The discovery of adiA expression in S. boydii serotype 18 by use of an acidified minimal medium challenge and arginine decarboxylase biochemical assay is significant because arginine decarboxylase activity was thought to be unique to E. coli. Sequencing of the rpoS gene from the S. boydii outbreak strain indicates that it is 99% conserved compared with the E. coli K-12 rpoS gene and plays a vital role in survival under acidic conditions.

Shigella is a foodborne pathogen that causes shigellosis. Clinical signs include bloody diarrhea, fever, abdominal cramps, and inflammation of intestinal mucosa and can be complicated by dehydration, seizures, hemolytic uremic syndrome (8), and Reiter’s syndrome (5). Imported foods from developing countries have been a concern because Shigella is more commonly found in these areas (11, 26).

There are four species of Shigella: S. dysenteriae, S. flexneri, S. boydii, and S. sonnei. These species are divided into subgroups A, B, C, and D, respectively. S. dysenteriae causes the most severe symptoms, S. boydii and S. flexneri produce mild to severe symptoms, and S. sonnei brings about mild symptoms. Although S. dysenteriae is more commonly associated with person-to-person transmission, S. flexneri, S. boydii, and S. sonnei are causative agents of foodborne illness brought about by improper food handling. The incubation period for shigellosis is 12 to 50 h. Mild symptoms of shigellosis can last for approximately 4 days, and severe symptoms can last for 10 to 14 days. Persons that consume foods that are contaminated with Shigella have a great risk of contracting infection because the infectious dose is very low. As few as 10 to 500 ingested cells can cause illness (17).

S. flexneri and S. sonnei can survive at a low pH for several hours and in acidic foods for extended periods (4, 20, 40, 42). Adaptation of cells in glucose or mild acid prior to introduction into an acidic environment can enhance survival compared with that of cells that are not adapted (16, 33, 40).

Acid stress response mechanisms have been studied extensively in Escherichia coli isolates and in S. flexneri, including sigma S (rpoS gene) (32, 35) and amino acid decarboxylases (3, 10, 22, 24, 25, 38). Sigma S is a global regulator of as many as 40 genes (21, 27), and some of these genes are involved in the acid stress response (41). Other characteristics of sigma S include complete activation during late-exponential- and stationary-phase growth (2, 34) and induction during nutrient limitation, especially during stationary phase (23). Amino acid decarboxylases such as the biodegradative arginine decarboxylase (adiA gene) and glutamate decarboxylase (gadCB gene) have been studied in E. coli (24, 25, 29). The mechanisms of action of amino acid decarboxylases are similar because the amino acid is taken into the cell, a proton (H+) is used in the decarboxylation reaction, the decarboxylation product (arginine → agmatine and glutamic acid → gamma-amino butyric acid) is taken out of the cell through an antiporter, and in this energy is stored to maintain the cell’s internal pH during external acid stress conditions (36). These genes may play a role in the survival of S. boydii under acidic conditions.

A strain of S. boydii 18 was implicated in a foodborne outbreak on 25 March 1998 at a restaurant in Chicago, Ill.

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The Chicago Department of Public Health (CDPH) investigated the incident and found that the pathogen was associated with the bean salad, which was most likely contaminated from the parsley or cilantro ingredients that were imported from Mexico. This outbreak strain is designated S. boydii CDPH serotype 18. The S. boydii outbreak strain survived better over time in bean salad at pH 4.0 to 5.0 than did the S. boydii ATCC 35966 strain (1). Other than this preliminary study, the acid tolerance characteristics of S. boydii have not been studied. In the present study, the acid survival of the S. boydii CDPH outbreak isolate was compared with that of other isolates of Shigella and E. coli that are known to survive in acidic foods and have the ability to pass the gastric barrier. The acid tolerance genes adiA and rpoS of S. boydii were also examined to determine how this foodborne pathogen is able to survive acidic conditions.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study were S. boydii 18 CDPH implicated in an outbreak involving bean salad, S. boydii 18 ATCC 35966 (a human isolate from the American Type Culture Collection), S. flexneri 3136 (John Foster laboratory, University of South Alabama, Mobile), E. coli O157:H7 dd8872 and E. coli dd8872 (Carl Batt laboratory, Cornell University, Ithaca, N.Y.), and E. coli K-12 from our laboratory collection.

Growth conditions. Strains were inoculated (1:100 dilution) into tryptic soy broth (TSB) or Luria-Bertani (LB) broth (Becton Dickinson). This assay was carried out at 37°C with shaking (200 rpm). Samples were taken at time 0, 24, and 48 h and spread plated in duplicate on tryptic soy agar (TSA) or LB agar. Cells were enumerated after 48 h of incubation. Each experiment was performed twice with similar results. The detection limit was set at ≥25 cells per plate.

Acid challenge assay in complex media. To compare differences in acid survival, an acid challenge in acidified TSB or LB were performed. TSB or LB was acidified using glacial acetic acid (Fisher Scientific, Fair Lawn, N.J.) to pH 4.5. Late-exponential-phase cultures were diluted to an optical density (wavelength 600 nm) of 1.5, inoculated (1:100 dilution) into the acidified medium, and incubated at 37°C with shaking (200 rpm). Samples were taken at time 0, 24, and 48 h and spread plated in duplicate on tryptic soy agar (TSA) or LB agar. Cells were enumerated after incubation at 37°C for 48 h to account for injured cells. Cells were spread plated in duplicate, and each experiment was performed twice. The detection limit was set at ≥25 cells per plate (plates containing <25 cells per plate were not counted).

Acid challenge assay in minimal media containing amino acids. Acid challenge in minimal medium was performed using an assay described by Lin et al. (24). This acid challenge was used to determine whether differences in acid survival could be detected in the presence of an amino acid of interest. Chemicals and reagents used were obtained from Sigma (St. Louis, Mo.) unless otherwise specified. Stationary-phase cultures were inoculated into LB containing 100 mM MES (morpholineethanesulfonic acid) at pH 5.5, LB with 100 mM MOPS (morpholinepropane-sulfonic acid) at pH 8.0, or LB with MES and 0.4% glucose at pH 5.5. Adapted cultures were grown to stationary phase. Cultures grown in only LB + MOPS and LB + MES were inoculated into prewarmed acidified LB at pH 2.5 using HCl. Cultures adapted to LB + MES + glucose were inoculated into prewarmed M9 minimal salts medium (Becton Dickinson) prepared according to the manufacturer's protocol and acidified to pH 2.5 using HCl. Nicotinamide (10⁻² M) was added to minimal medium used for S. flexneri. Acidified M9 minimal salts media contained no amino acids, 0.012% arginine, or 0.012% glutamic acid. Acid challenge was carried out at 37°C with shaking (250 rpm) for 2 h. This particular pH and time was used to mimic conditions bacteria would encounter in the human gastric system. Cells were spread plated on LB agar in duplicate. Plates were incubated at 37°C, and viable counts were determined after 48 h of incubation. Each experiment was performed twice with similar results. The reproducibility of the data was within 50% of the survival values (if the percent survival is 50, then values were between 25 and 75%). Percent survival was calculated as a percentage of the final viable count (at 2 h) divided by the initial viable count (at 0 h).

Arginine decarboxylase biochemical assay. Arginine decarboxylase activity was evaluated using Moeller decarboxylase medium containing arginine (Becton Dickinson). This assay was used to confirm arginine decarboxylase activity results found in the acidified minimal medium acid challenge. A 24-h culture was inoculated (1:100 dilution), overlaid with sterile mineral oil, and incubated at 37°C for 4 days. This medium contains a pH indicator bromocresol purple. The medium is purple when arginine decarboxylase activity is present and yellow when it is not.

PCR amplification and nucleotide sequencing of adiA and rpoS. To compare the sequences of S. boydii isolates, samples were analyzed by PCR amplification and nucleotide sequencing. Genomic DNA was isolated using a genomic DNA purification kit (Wizard, Promega, Madison, Wis.) according to the manufacturer’s protocols. Primers used for genomic DNA amplification of the S. boydii adiA and rpoS genes were designed based on the E. coli K-12 adiA gene (GenBank accession no. M93362) and E. coli rpoS gene (GenBank accession no. D13548). For the adiA gene, the primers used for amplification of the arginine decarboxylase gene were 5′-AGCCGTCGATAAAGCAG-3′ and 5′-CTTTC CCATTCACCTTATC-3′. For the rpoS gene, the primers used in the PCR amplification were 5′-CGGAACCAGGCTTTTGC TTGAATG-3′ and 5′-ATGGGCATCGGACCTTTATTGTG-3′.
FIGURE 2. Acid challenge of late-exponential-phase E. coli O157:H7 dd8872, E. coli O157:H7 dd642, S. flexneri 3136, S. boydii CDPH, and S. boydii ATCC 35966 in LB containing acetic acid at pH 4.5. Colonies on TSA aerobic plates were counted after 48 h. The experiment was repeated twice with similar results. The detection limit was set at ≥25 cells per plate.

Both sets of primers bind to 3' and 5' flanking regions at the end of each gene. Amplification and sequencing was performed on the entire coding region of each gene. The final concentrations of all PCR reagents were 1.5 mM MgCl₂, 1 mM dNTPs, 2 µM forward primer, 2 µM reverse primer, 1 × PCR buffer, 2.5 U Taq polymerase (Invitrogen, Carlsbad, Calif.), and 0.5 µl of genomic DNA for each 50-µl reaction. Thermocycling parameters for amplification of the adiA gene were 5 min at 94°C for initial denaturation, 24 cycles of 1 min at 94°C for denaturing, 1 min at 54.6°C for annealing, and 3 min at 72°C for extension, and 7 min at 72°C for the final extension. The parameters for amplification of the rpoS gene were 5 min at 94°C for initial denaturation, 29 cycles of 30 s at 94°C for denaturing, 45 s at 67°C for annealing, and 30 s 72°C for extension, and 7 min at 72°C for final extension. PCR products were visualized using 0.8% agarose gel electrophoresis containing 1× TAE (40 mM Tris-HCl, pH 8.0, 20 mM acetic acid, 2 mM EDTA, and 12.5 mM magnesium acetate) and stained with ethidium bromide. Nucleotide sequencing was carried out at the W. M. Keck Center for Comparative and Functional Genomics (Urbana, Ill.). Resulting sequences were analyzed using Lasergene (DNASTAR, Inc., Madison, Wis.), and alignments were made using ClustalW (http://www.ebi.ac.uk/clustalw/). Sequencing was performed twice with two independent PCRs.

Antibiotics used. For testing antibiotic resistance of S. boydii strains, tetracycline (15 mg/ml), ampicillin (100 mg/ml), streptomycin (100 mg/ml), erythromycin (35 mg/ml), penicillin (250 mg/ml), kanamycin (30 mg/ml), chloramphenicol (20 mg/ml), or rifampicin (50 mg/ml) were used in LB agar. An overnight culture was grown at 37°C with shaking (200 rpm). Cells were streak plated onto LB agar containing the antibiotic and incubated at 37°C for 24 h.

Statistical analysis. Statistical analyses were performed using a general linear model (α = 0.05) of SAS (SAS Institute, Inc., Cary, N.C.).

RESULTS AND DISCUSSION

Acid challenge in complex media. In this acid tolerance study, the S. boydii foodborne outbreak strain was compared with other related enteric bacteria such as environmental samples of E. coli O157:H7 that are known to be acid tolerant (7, 15, 28). At pH 4.5, the acid challenge of various strains in acidified TSB revealed differences in survival. The initial cell count of all strains was an average of 2 × 10⁷ CFU/ml. By 24 h, S. boydii ATCC 35966 had
TABLE 1. Percent survival of various bacterial strains in acidified LB or acidified M9 (no amino acid, arginine, or glutamic acid) media at pH 2.5

<table>
<thead>
<tr>
<th>Strain</th>
<th>Adaptation prior to challenge</th>
<th>Acid challenge medium</th>
<th>% survival*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. flexneri 3136</td>
<td>LB + MOPS, pH 8.0</td>
<td>LB + HCl, pH 2.5</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>LB + MES, pH 5.5</td>
<td>LB + HCl, pH 2.5</td>
<td>29.5</td>
</tr>
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<td>LB + 1% glucose + MES, pH 5.5</td>
<td>M9 + HCl, pH 2.5</td>
<td>4.0</td>
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<tr>
<td></td>
<td></td>
<td>M9 + arginine + HCl, pH 2.5</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M9 + glutamic acid + HCl, pH 2.5</td>
<td>66.4</td>
</tr>
<tr>
<td>S. boydii CDPH</td>
<td>LB + MOPS, pH 8.0</td>
<td>LB + HCl, pH 2.5</td>
<td>9.4</td>
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<td>LB + MES, pH 5.5</td>
<td>LB + HCl, pH 2.5</td>
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<td></td>
<td>LB + 1% glucose + MES, pH 5.5</td>
<td>M9 + HCl, pH 2.5</td>
<td>4.5</td>
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<td></td>
<td></td>
<td>M9 + arginine + HCl, pH 2.5</td>
<td>67.3</td>
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<td>M9 + glutamic acid + HCl, pH 2.5</td>
<td>47.7</td>
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<td>LB + HCl, pH 2.5</td>
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<td>M9 + arginine + HCl, pH 2.5</td>
<td>48.1</td>
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<td>M9 + glutamic acid + HCl, pH 2.5</td>
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<td>E. coli O157:H7 dd8872</td>
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<td>E. coli O157:H7 dd642</td>
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<td>M9 + HCl, pH 2.5</td>
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<td>M9 + arginine + HCl, pH 2.5</td>
<td>80.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M9 + glutamic acid + HCl, pH 2.5</td>
<td>0.0026</td>
</tr>
</tbody>
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*Percent survival is expressed as a percentage of the final colony count compared with the initial colony count: ≥10% survival is acid resistant, 0.1 to 9% is moderately acid resistant, and <0.1% is acid sensitive. The reproducibility of the data was within 50% of the percent survival values.

decreased to less than 10 log CFU/ml (below the detection limit), which represents approximately seven orders of magnitude reduction. S. boydii CDPH, E. coli O157:H7 dd642, and E. coli K-12 had similar survival characteristics in TSB at pH 4.5 ($P = 0.4562$) (Fig. 1). S. boydii CDPH was considerably more acid tolerant than S. boydii ATCC 35966; S. boydii CDPH was detected at 24 and 48 h, whereas S. boydii ATCC was not detected at those time points. Also at the 24-h time point, S. boydii CDPH was more acid tolerant than S. boydii ATCC 35966 ($P = 0.0001$) (Fig. 2). These data demonstrate that acid tolerance can vary considerably between S. boydii, E. coli K-12, and E. coli O157:H7, and large variations in survival are possible among S. boydii type 18 strains.

**Acid challenge in acidified minimal media using amino acids.** Acid resistance of S. flexneri 3136, S. boydii CDPH, S. boydii ATCC 35966, E. coli O157:H7 dd8872, and E. coli O157:H7 dd642 was measured by using acidified minimal media at pH 2.5 (Table 1). This assay was

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**FIGURE 4.** (a) Agarose gel electrophoresis of adA PCR amplification using a genomic DNA template. Lane 1, 1-kb DNA ladder; lane 2, S. flexneri 3136; lane 3, S. boydii CDPH; lane 4, S. boydii ATCC 35966; lane 5, E. coli K-12. (b) Agarose gel electrophoresis of rpoS PCR amplification using a genomic DNA template. Lane 1, 1-kb DNA ladder; lane 2, E. coli K-12; lane 3, S. flexneri 3136; lane 4, S. boydii CDPH; lane 5, S. boydii ATCC 35966.
performed twice with similar results. E. coli O157:H7 dd8872 and E. coli O157:H7 dd642 were acid inducible; the percent survival of the MES-adapted (pH 5.5) strains was higher than that of the same strains adapted to MOPS (pH 8.0) \( (P = 0.0018 \text{ for } E. coli \ O157:H7 \ dd8872; \ P = 0.0187 \text{ for } E. coli \ O157:H7 \ dd642) \). Similar to previous minimal medium challenges done by Lin et al. (24), the MES-adapted S. flexneri 3136 appeared to survive better than did the MOPS-adapted cells, although there was not a significant difference. These results are consistent with the observation that S. flexneri and E. coli O157:H7 have acid-inducible amino acid decarboxylase systems (19, 24). S. boydii CDPH and S. boydii ATCC 35966 were not acid inducible when compared with the other enteric bacteria tested in the same assay; the MOPS-adapted S. boydii strains were able to survive better than MES-adapted strains in LB at pH 2.5 for 2 h in the same acid challenge conditions. In acidified M9 containing no amino acids, all strains had less than 10% survival, which was expected. The survival percentages for S. flexneri 3136, S. boydii CDPH, S. boydii ATCC 35966, E. coli O157:H7 dd8872, and E. coli O157:H7 dd642 in acidified M9 containing no amino acids were 4, 4.5, 2.5, 1.4, and 0.068%, respectively. S. flexneri 3136 was not able to survive (4%) in acidified M9 containing arginine, whereas S. boydii 3136 was not able to survive (4%) in acidified M9 containing arginine because S. boydii strains in general have arginine decarboxylase activity. In acidified M9 containing glutamic acid, all strains were able to survive except for E. coli O157:H7 dd642, which had 0.0026% survival. Based on the results obtained from this acidified minimal medium assay, there may be a defect in glutamate decarboxylase activity in E. coli O157:H7 dd642. E. coli O157: H7 dd642 was not able to survive as long as E. coli O157: H7 dd8872 in acidified TSB and LB at time point 48 h (Figs. 1 and 2).

S. boydii strains were able to survive at pH 2.5 for 2 h in minimal medium containing arginine or glutamic acid. The results of the acidified minimal media assay indicate that S. boydii CDPH and S. boydii ATCC 35966 have an arginine decarboxylase, which was previously thought to be an acid resistance system unique to E. coli (19). Results obtained in Moeller decarboxylase broth containing arginine confirm that the S. boydii strains have arginine decarboxylase activity. The adiA gene of E. coli K-12 has been sequenced (38), and there is 98% nucleotide sequence conservation between it and the adiA gene associated with S. boydii CDPH. The arginine decarboxylase associated with S. boydii 18 strains apparently is not acid inducible, whereas that associated with E. coli K-12 and E. coli O157:H7 strains is acid inducible (19, 38). Other genes in E. coli contribute to acid-induced tolerance (39). However, the role of these genes has not been examined in Shigella.

**PCR amplification and nucleotide sequencing of adiA and rpoS.** Agarose gel electrophoresis of amplified adiA DNA revealed a 3-kb gene in S. flexneri 3136, E. coli K-12, and both S. boydii strains examined in this study. However, S. flexneri 3136 arginine decarboxylase activity was not found. Because the S. boydii arginine decarboxylase was not acid inducible, the strains were tested in Moeller decarboxylase broth containing arginine. Cultures of S. boydii strains were purple, i.e., positive for arginine decarboxylase activity. S. flexneri 3136 cultures were yellow (negative) and were used as a negative control.

The adiA gene of S. boydii CDPH was sequenced (GenBank accession no. AF231474) and analyzed using BLAST. This gene was most similar (98%) to the genes of E. coli K-12 MG1655, E. coli K-12, and E. coli O157:H7 (GenBank accession nos. U14003, AE000484 and U00096, M93362, and AP00268 and BA00007). An alignment of E. coli K-12 (GenBank accession no. U02384) and S. boydii CDPH adiA is shown in Figure 3.

PCR amplification of the 1-kb rpoS gene derived from E. coli K-12, S. boydii CDPH, S. boydii ATCC 35966, and S. flexneri 3136 suggests that the S. boydii rpoS is similar to that of E. coli K-12 (Fig. 4).

Sequencing and BLAST analysis demonstrated that the rpoS gene of S. boydii CDPH (GenBank accession no. AF231472) is 99% conserved compared with several E.
coli K-12 rpoS sequences (GenBank accession nos. AE000358, U29579, and D13548). Comparative analysis of the S. boydii CDPH rpoS and that of S. flexneri 3136 (GenBank accession no. U00119) revealed 97% identity. Observed differences in the nucleotide sequences were found scattered throughout the gene.

Comparison of the sequence of the rpoS gene of S. boydii CDPH with that of S. boydii ATCC 35966 (GenBank accession no. Y231473) revealed one residue alteration at amino acid 33 (Fig. 5). This change from T to G alters the codon from TTA (leucine) to TGA (stop). The alteration is significant because TGA is a stop codon that occurs early in the S. boydii ATCC 35966 rpoS gene. Therefore, the S. boydii ATCC 35966 rpoS gene probably is not expressed.

The rpoS sequences of E. coli strains such as E. coli O157:H7 and E. coli K-12 have been compared, and several amino acid differences have been found in some strains whereas other E. coli strains have identical rpoS sequences (18). Shigella is closely related to E. coli; the DNA homology between the two taxa is 70 to 100% (9). This similarity is apparent when comparing the S. boydii rpoS and E. coli K-12 rpoS nucleotide sequences.

The results of this study indicate that S. boydii 18 strains have both an arginine decarboxylase and a glutamate decarboxylase. However, the difference in acid tolerance between E. coli O157:H7 strains and S. boydii strains is still apparent; E. coli O157:H7 dd8872 is significantly more acid tolerant than is the S. boydii CDPH outbreak strain. These results suggest that there is possibly another mechanism responsible for the observed difference in acid resistance between E. coli and Shigella. Lysine decarboxylase may play an important role in acid resistance, and this acid resistance system is said to be present in E. coli spp. (29–31). Other acid stress response mechanisms and environmental factors known to enhance survival of E. coli in acidic conditions have not been studied in S. boydii, and these mechanisms may also contribute to the survival of S. boydii in acidic conditions (6, 13, 33, 42).

Chugh et al. (14) found that approximately 77% of the clinical isolates of Shigella examined were resistant to three or more antibiotics and 58% were resistant to five or more antibiotics. S. boydii CDPH on LB agar also is resistant to a number of antibiotics, including tetracycline, ampicillin, streptomycin, erythromycin, and penicillin. Infections caused by Shigella strains resistant to multiple antibiotics make treatment difficult. The treatment for shigellosis is fluid replacement. Antibiotics are either not necessary or not desirable because so many strains of Shigella have become resistant to antibiotics.

The antibiotic profile of the S. boydii outbreak strain in combination with acid tolerance and resistance mechanisms make it a potentially dangerous pathogen when present in acidic foods. This results of the present research indicate that the S. boydii 18 foodborne outbreak strain is acid resistant in acidified minimal medium containing arginine or glutamic acid, which further suggests that acid tolerance genes associated with the S. boydii 18 outbreak strain may allow the pathogen to bypass the gastric barrier; therefore, only a small number of cells may be needed to cause illness.

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