

Research Note

Isolation of *Salmonella* Typhimurium from Outbreak-Associated Cake MixGUODONG ZHANG,^{1,2} LI MA,^{1,2} NEHAL PATEL,² BALA SWAMINATHAN,² STEPHANIE WEDEL,³ AND MICHAEL P. DOYLE^{1*}¹Center for Food Safety, University of Georgia, 1109 Experiment Street, Griffin, Georgia 30223; ²Centers for Disease Control and Prevention, Atlanta, Georgia 30333; and ³Minnesota Department of Health, St. Paul, Minnesota 55164, USA

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ABSTRACT

During May and June of 2005, 26 persons in several states were infected by a single strain (isolates indistinguishable by pulsed-field gel electrophoresis) of *Salmonella enterica* serotype Typhimurium after eating cake batter ice cream. The cake mix used to prepare the cake batter in the ice cream was implicated by epidemiologic investigation as the source of *Salmonella* contamination. Initial tests did not detect *Salmonella* in cake mix collected during the outbreak investigation. The objective of this study was to evaluate different procedures to isolate *Salmonella* from the implicated cake mix, cake, and ice cream. All outbreak-associated food samples (14 samples) were collected during the outbreak investigation by health departments of several of the states involved. Different combinations of *Salmonella* isolation procedures, including sample size, preenrichment broth, enrichment broth, enrichment temperature, and isolation medium, were used. *Salmonella* Typhimurium was isolated from two cake mix samples; the food isolates were indistinguishable from the outbreak pattern by pulsed-field gel electrophoresis subtyping. Universal preenrichment broth was substantially better than was lactose broth for preenrichment, and tetrathionate broth was better than was Rappaport-Vassiliadis broth for isolating *Salmonella* from the two positive cake mix samples. Although more typical *Salmonella* colonies were observed on plates from enrichment cultures grown at 35°C, more confirmed *Salmonella* isolates were obtained from plates of enrichment cultures grown at 42°C. Brilliant green agar, xylose lysine tergitol 4 agar, xylose lysine desoxycholate agar, Hektoen enteric agar, and bismuth sulfite agar plates were equally effective in isolating *Salmonella* from cake mix. The best combination of preenrichment-enrichment conditions for isolating the outbreak strain of *Salmonella* was preenrichment of cake mix samples in universal preenrichment broth at 35°C for 24 h, followed by enrichment in tetrathionate broth at 42°C for 24 h.

An estimated 1.4 million cases of foodborne salmonellosis occur annually in the United States (11). Outbreaks of foodborne salmonellosis are widespread throughout the world (2, 3, 5–7, 10–13, 19). During May and June 2005, 26 persons in Minnesota, Washington, Oregon, Virginia, Ohio, California, Illinois, Maryland, Michigan, Florida, and Pennsylvania were infected by the same pulsed-field gel electrophoresis (PFGE) subtype of *Salmonella enterica* serotype Typhimurium, with 25 reported to have eaten cake batter ice cream from Creamery A stores (chain A) (19). The U.S. Food and Drug Administration (FDA) issued a warning on 1 July 2005 that products containing “cake batter” ice cream sold at chain A throughout the country may be associated with an outbreak of *Salmonella* Typhimurium in several states (17, 19). On a voluntary basis, chain A promptly removed all cake batter products from all of its stores. Cake mix used in cake batter ice cream was implicated as the vehicle. Ice cream and cake mix samples associated with the outbreak were analyzed by several different laboratories, and *Salmonella* was not detected. The objective of this study was to evaluate the efficacy of sev-

eral different parameters used to isolate *Salmonella* from foods in attempting to isolate the outbreak strain of *Salmonella* from outbreak-associated cake mix and ice cream samples.

MATERIALS AND METHODS

Food samples. All food samples were collected in July 2006 by four health departments of states involved in the outbreak. They included five cake mixes from Minnesota; two cake mixes from Washington; one cake mix from Oregon; and one cake mix, two containers of cake batter ice cream, and three different cakes from Ohio. Samples were stored at –23°C upon receipt at the Center for Food Safety, University of Georgia, until used for microbiologic analysis in September and October 2006.

Isolation of *Salmonella*. Several different media and conditions either used by government food regulatory agencies or previously published as successful for isolating or detecting *Salmonella* species in foods were employed. Preenrichment broths included universal preenrichment broth (Difco, Becton Dickinson, Sparks, Md.) and lactose broth (Difco, Becton Dickinson). Two sample sizes were evaluated, with 25 g of food samples being added to 225 ml of preenrichment broth or 100 g to 900 ml preenrichment broth. The 100-g sample was used only with universal preenrichment broth because an insufficient amount of sample was available to enable testing a second 100-g portion of most of the

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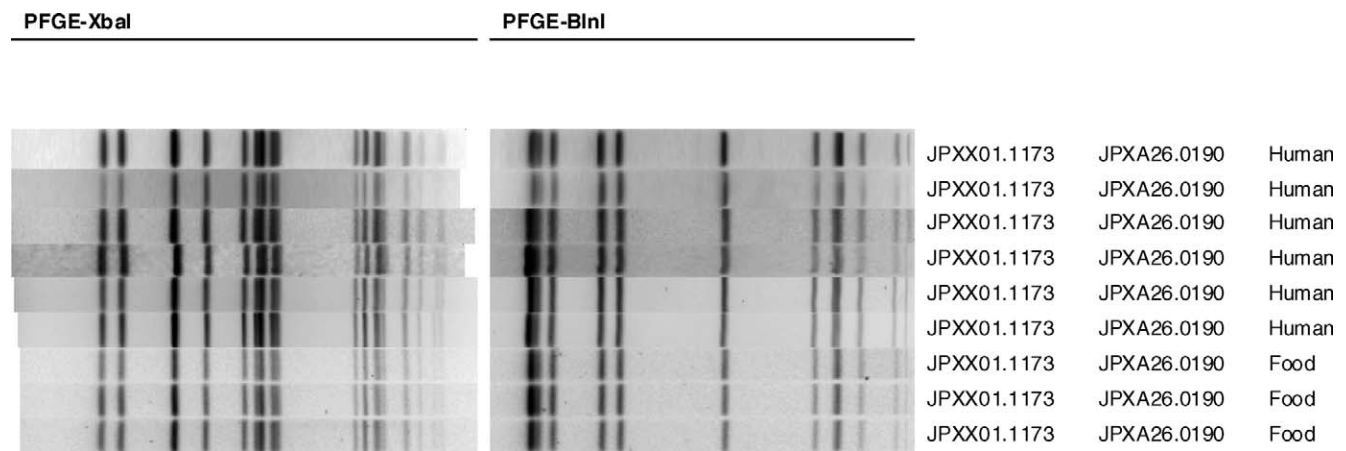


FIGURE 1. Normalized pulsed-field gel electrophoresis patterns of XbaI- and BlnI-digested genomic DNA of *Salmonella* Typhimurium isolates from clinical and food isolates associated with the outbreak. Pattern numbers shown to the right of each pattern are designated according to standardized PulseNet pattern naming system. JPX is the code for *Salmonella* Typhimurium; X01, XbaI restriction enzyme; A26, BlnI restriction enzyme. The pattern numbers are sequentially assigned to each unique pattern in the PulseNet database. Same number in the pulsed-field gel electrophoresis pattern designation indicates that the patterns are indistinguishable from each other.

samples. The 1-liter preenrichment mixes could not be homogenized in Laboratory Blender Stomacher 400 (Seward, London, UK) in our laboratory; therefore, all samples were mixed well by shaking and swirling vigorously by hand for 1 min, followed by stirring for 10 min on a stirrer at room temperature. Samples were subsequently incubated at 35°C for 20 to 24 h. After preenrichment, 0.5 ml of preenrichment broth was added to 10 ml of tetrathionate broth (Hajna) (TT; Difco, Becton Dickinson) and 0.1 ml into 10 ml of Rappaport-Vassiliadis broth (RV; Difco, Becton Dickinson) for selective enrichment. Selective enrichment cultures were incubated at 35 or 42°C for 20 to 24 h in an air incubator. Subsequent to selective enrichment, 10 µl of selective enrichment culture was streaked, and 100 µl was plated onto brilliant green agar (BGA; Difco, Becton Dickinson), xylose lysine tergitol 4 agar (XLT4; Difco, Becton Dickinson), xylose lysine desoxycholate agar (XLD; Difco, Becton Dickinson), Hektoen enteric agar (HE; Difco, Becton Dickinson), and bismuth sulfite agar (BSA; Difco, Becton Dickinson) plates for each culture. All plates were incubated at 35°C for 24 h afterward. One typical and atypical *Salmonella* colony was selected from each plate for identification.

Identification of *Salmonella*. Suspected *Salmonella* colonies were identified using the Microgen *Salmonella* Latex Agglutination kit (Microgen Bioproducts, Ltd., Surrey, UK), the API 20E kit (bioMérieux, Inc, Hazelwood, Mo.), and the BAX Detection System (DuPont Qualicon, Wilmington, Del.) according to manufacturers' instructions. In addition, some isolates were confirmed by 16S rRNA gene sequencing using the MicroSeq Microbial Identification System (MIDI LABS, Newark, Del.). Serotyping of *Salmonella* was done in the Centers for Disease Control and Prevention, according to Brenner and McWhorter-Murlin (4).

PFGE subtyping of *Salmonella* Typhimurium isolates. Food isolates of *Salmonella* Typhimurium were subtyped according to the PulseNet standardized protocol (14) and were compared with the cake mix outbreak-associated clinical isolates in the PulseNet National *Salmonella* Database, using standard PulseNet matching parameters (8).

RESULTS

Of the 14 cake mix, cake, and ice cream samples tested, two cake mix samples were positive for *Salmonella* Ty-

phimurium. Isolates from both *Salmonella*-positive cake mix samples were indistinguishable from the outbreak strain by PFGE subtyping (Fig. 1). One sample obtained from Minnesota was received in a plastic Ziploc bag, whereas the other, obtained from the State of Washington, was received in an opened, original package. *Salmonella* was not isolated from the cake and ice cream samples.

Results of the efficacy of the different *Salmonella* isolation conditions evaluated in obtaining typical *Salmonella* colonies or no growth on selective agar plates are listed in Table 1. Preenrichment in the universal preenrichment broth yielded many more selective differential agar plates with typical *Salmonella* colonies than did preenrichment in lactose broth. There was no significant difference between the numbers of typical *Salmonella* colonies on plates from 25- or 100-g preenrichment cultures. Substantially more plates with typical *Salmonella* colonies were obtained from cultures enriched in TT broth than from RV broth regardless of preenrichment broth and incubation temperature, and an enrichment temperature of 35°C produced many more plates with typical *Salmonella* colonies than did 42°C. There was very little bacterial growth on XLD and XLT4 plates, whereas an abundance of typical *Salmonella* colonies grew on BGA, HE, and BSA plates.

A comparison of the isolation conditions for obtaining typical *Salmonella* colonies on selective isolation media plates from the two *Salmonella*-positive cake mix samples is shown in Table 2. For cake mix sample box 1, there were 21 confirmed *Salmonella* isolates from universal preenrichment broth compared with none from lactose broth. Similarly, for cake mix sample M-05-1440, there were 18 confirmed *Salmonella* isolates from universal preenrichment broth compared with 3 from lactose broth. As for enrichment broths, 18 confirmed *Salmonella* colonies among 33 typical *Salmonella* colonies were obtained from box 1, using TT broth for enrichment, whereas only 3 confirmed colonies were obtained from 23 suspect colonies with RV broth. In contrast, comparable results were obtained with

TABLE 1. Number of selective agar plates with typical *Salmonella* colonies and number of plates with no growth from outbreak-associated samples under different *Salmonella* isolation conditions^a

| Enrichment broth | Enrichment temp (°C) | Media for isolation | 25-g sample/225 ml of lactose broth | | 25-g sample/225 ml of universal preenrichment broth | | 100-g sample/900 ml of universal preenrichment broth | |
|------------------|----------------------|---------------------|---|--|---|--|---|--|
| | | | No. of plates with typical <i>Salmonella</i> colonies/total no. of plates (%) | No. of plates with no growth/total no. of plates (%) | No. of plates with typical <i>Salmonella</i> colonies/total no. of plates (%) | No. of plates with no growth/total no. of plates (%) | No. of plates with typical <i>Salmonella</i> colonies/total no. of plates (%) | No. of plates with no growth/total no. of plates (%) |
| TT | 42 | BGA | 28/72 (39) | 12/72 (17) | 56/72 (78) | 0/72 (0) | 56/64 (88) | 0/64 (0) |
| | | XLT4 | 0/72 (0) | 60/72 (83) | 0/72 (0) | 40/72 (56) | 4/64 (6) | 16/64 (25) |
| | | XLD | 0/72 (0) | 44/72 (61) | 6/72 (8) | 0/72 (0) | 4/64 (6) | 4/64 (6) |
| | | HE | 35/72 (49) | 32/72 (44) | 63/72 (88) | 0/72 (0) | 42/64 (66) | 4/64 (6) |
| | | BSA | 40/72 (56) | 20/72 (28) | 64/72 (89) | 4/72 (6) | 55/64 (86) | 4/64 (6) |
| | 35 | BGA | 40/72 (56) | 0/72 (0) | 60/72 (83) | 0/72 (0) | 53/64 (83) | 0/64 (0) |
| | | XLT4 | 8/72 (11) | 8/72 (11) | 0/72 (0) | 0/72 (0) | 4/64 (6) | 0/64 (0) |
| | | XLD | 4/72 (6) | 8/72 (11) | 4/72 (6) | 0/72 (0) | 4/64 (6) | 0/64 (0) |
| | | HE | 48/72 (67) | 4/72 (6) | 64/72 (89) | 0/72 (0) | 52/64 (81) | 0/64 (0) |
| | | BSA | 66/72 (92) | 4/72 (6) | 72/72 (100) | 0/72 (0) | 64/64 (100) | 0/64 (0) |
| RV | 42 | BGA | 12/72 (17) | 28/72 (39) | 24/72 (33) | 28/72 (39) | 40/64 (63) | 0/64 (0) |
| | | XLT4 | 0/72 (0) | 64/72 (89) | 0/72 (0) | 44/72 (61) | 4/64 (6) | 52/64 (81) |
| | | XLD | 0/72 (0) | 52/72 (72) | 4/72 (6) | 32/72 (44) | 4/64 (6) | 32/64 (50) |
| | | HE | 18/72 (25) | 48/72 (67) | 26/72 (36) | 32/72 (44) | 21/64 (33) | 28/64 (44) |
| | | BSA | 36/72 (50) | 28/72 (39) | 37/72 (51) | 24/72 (33) | 62/64 (97) | 0/64 (0) |
| | 35 | BGA | 40/72 (56) | 0/72 (0) | 50/72 (69) | 0/72 (0) | 52/64 (81) | 0/64 (0) |
| | | XLT4 | 0/72 (0) | 8/72 (11) | 4/72 (6) | 4/72 (6) | 12/64 (19) | 4/64 (6) |
| | | XLD | 0/72 (0) | 0/72 (0) | 4/72 (6) | 0/72 (0) | 4/64 (6) | 12/64 (19) |
| | | HE | 40/72 (56) | 0/72 (0) | 46/72 (64) | 0/72 (0) | 40/64 (63) | 4/64 (6) |
| | | BSA | 72/72 (100) | 0/72 (0) | 66/72 (92) | 0/72 (0) | 64/64 (100) | 0/64 (0) |

^a TT, tetrathionate broth; BGA, brilliant green agar; XLT4, xylose lysine tergitol 4 agar; XLD, xylose lysine desoxycholate agar; HE, Hektoen enteric agar; BSA, Bismuth sulfite agar; RV, Rappaport-Vassiliadis broth.

TT and RV broths with sample M-05-1440. Enrichment at 42°C produced more confirmed *Salmonella* colonies than were produced at 35°C; however, the results were not dramatically different. For cake mix sample M-05-1440, about 50% of the typical *Salmonella* colonies selected was confirmed to be *Salmonella* across the five selective isolation media BGA, XLD, XLT4, HE, and BSA. For cake mix sample box 1, all typical *Salmonella* colonies (eight) from XLD and XLT4 were confirmed to be *Salmonella*, and 40% or less of the typical *Salmonella* colonies from the three other plates were confirmed *Salmonella* positive. Preenrichment sample size was a factor but was sample dependent, with all confirmed *Salmonella* colonies from sample box 1 obtained from the 100-g preenrichment, and sample M-05-1440 from the 25-g preenrichment.

DISCUSSION

Cake mix contains several raw ingredients, and is intended to be reconstituted and baked sufficiently to kill vegetative cells of pathogens that may be present in flour or other ingredients of cake mix. Therefore, microbes in cake mix usually are not considered a public health concern. Because raw cake mix used in cake batter ice cream is not given a bactericidal treatment such as baking, pathogenic microbes in cake mix would contaminate the ice cream. All *Salmonella*-positive persons except one ate cake batter ice

cream in the outbreak associated with chain A; hence, the raw cake mix (primary ingredients included sugar, flour, soybean and cottonseed oil, baking soda, corn starch, propylene glycol monoesters of fatty acids, and egg white) as the main ingredient in cake batter ice cream became the suspected source of *Salmonella* (19).

As initial tests by several laboratories did not isolate any *Salmonella* from cake batter ice cream samples by the FDA *Bacteriological Analytical Manual* (16) or other *Salmonella* detection methods, we used a variety of *Salmonella* isolation conditions, including U.S. Department of Agriculture, Food Safety and Inspection Service (15) and FDA methods and others. Universal preenrichment broth and lactose broth were selected as preenrichment broths; TT and RV broths as enrichment broths; and BGA, XLT4, XLD, HE, and BSA as isolation media.

Universal preenrichment broth produced a higher percentage of plates with typical *Salmonella* colonies across the different isolation media studied and a lower percentage of plates without any colonies in comparison with lactose broth (Table 1). Among the 42 confirmed *Salmonella* colonies, 39 were from universal preenrichment broth (Table 2), indicating that universal preenrichment broth was considerably more effective for isolating *Salmonella* than was lactose broth. Hammack et al. (9) compared the effectiveness of lactose and universal preenrichment broths for re-

TABLE 2. Number of typical *Salmonella* colonies selected compared with number of confirmed *Salmonella* colonies from two *Salmonella* Typhimurium-positive outbreak-associated samples, using different *Salmonella* isolation conditions^a

| | Box 1, WA | | M-05-1440, MN | |
|-------------------------------|--|---|--|---|
| | No. of typical <i>Salmonella</i> colonies selected | No. of confirmed <i>Salmonella</i> isolates (%) | No. of typical <i>Salmonella</i> colonies selected | No. of confirmed <i>Salmonella</i> isolates (%) |
| Preenrichment sample size (g) | | | | |
| 100 | 32 | 21 (66) ^b | 9 | 0 (0) |
| 25 | 24 | 0 (0) | 30 | 21 (70) |
| Preenrichment broths | | | | |
| Universal preenrichment broth | 44 | 21 (48) | 29 | 18 (62) |
| Lactose broth | 12 | 0 (0) | 10 | 3 (30) |
| Enrichment broths | | | | |
| TT | 33 | 18 (55) | 25 | 12 (48) |
| RV | 23 | 3 (13) | 14 | 9 (64) |
| Enrichment temp (°C) | | | | |
| 35 | 32 | 7 (22) | 18 | 9 (50) |
| 42 | 24 | 14 (58) | 21 | 12 (57) |
| Media for isolation | | | | |
| BGA | 14 | 3 (21) | 8 | 4 (50) |
| XLT4 | 4 | 4 (100) | 7 | 4 (57) |
| XLD | 4 | 4 (100) | 8 | 5 (63) |
| HE | 15 | 6 (40) | 8 | 5 (63) |
| BSA | 19 | 4 (21) | 8 | 3 (38) |

^a TT, tetrathionate broth; RV, Rappaport-Vassiliadis broth; BGA, brilliant green agar; XLT4, xylose lysine tergitol 4 agar; XLD, xylose lysine desoxycholate agar; HE, Hektoen enteric agar; BSA, bismuth sulfite agar.

^b Percentage of confirmed *Salmonella* colonies from typical *Salmonella* colonies selected from selective media isolation plates.

covery of *Salmonella* from dairy foods. There was no significant difference between the two broths for isolating *Salmonella* from liquid whole milk, but universal preenrichment broth was significantly more productive for isolating *Salmonella* from lactic casein than was lactose broth. Although TT enrichment broth produced substantially more isolation plates with typical *Salmonella* colonies than RV broth, based on the number of confirmed *Salmonella* isolates from the two *Salmonella*-positive samples, TT broth was only slightly better than RV broth was. For sample M-05-1440, 12 confirmed *Salmonella* isolates among 29 suspect isolates were obtained from TT broth, whereas 9 confirmed *Salmonella* isolates among 14 suspect isolates were obtained from RV broth. For sample box 1, 18 confirmed *Salmonella* isolates among 33 typical *Salmonella* colonies were obtained from TT broth, whereas 3 confirmed *Salmonella* isolates among 23 typical *Salmonella* colonies were obtained from RV broth (Tables 1 and 2). Bailey et al. (1) reported that TT broth was better than selenite brilliant green broth and selenite cystine broth for isolating

Salmonella from fresh poultry and pork sausage, whereas selenite brilliant green broth was more productive than was TT broth for isolating *Salmonella* from cured chicken.

Enrichment temperature played an important role in suppressing non-*Salmonella*-like colonies. Although enrichment at 42°C produced substantially more plates without any colonies than did enrichment at 35°C (Table 1), a higher percentage of confirmed *Salmonella* colonies were obtained from typical colonies obtained from 42°C enrichment broths than from 35°C enrichment broths (Table 2). Enrichment at 42°C was better in isolating *Salmonella* from cake mix than was 35°C. This is in agreement with a previous report indicating that enrichment at 43°C was more effective than was 35°C for isolating *Salmonella* from fresh poultry, pork sausage, and cured chicken (1).

Among the five isolation media studied, XLT4 and XLD had much less bacterial growth than did BGA, HE, and BSA (Table 1). However, *Salmonella* isolates were obtained from all five media for the two positive samples (Table 2). XLT4 and XLD were more effective in inhibiting non-*Salmonella* bacterial growth than BGA, HE, and BSA were. Because all five media were effective in isolating *Salmonella* from cake mix, a larger, more comprehensive study is needed to determine which media are best for isolating salmonellae from cake mix. Seventeen Canadian federal, provincial, and public health laboratories conducted a comparative, collaborative study that evaluated a variety of commercial media, including BSA, HE, XLD, brilliant green sulfa agar, EF-18 agar, and Rambach agar for isolation of *Salmonella* (18). Their results revealed that EF-18 agar recovered the greatest number of *Salmonella* isolates. HE ranked second, with the other agars being comparable in their recovery of *Salmonella* spp. A comparison of brilliant green sulfa agar, modified lysine iron agar, and XLD with novobiocin for isolating *Salmonella* from fresh and cured meats revealed that modified lysine iron agar detected more positive samples than did the other two media. All three media performed adequately for differentiating salmonellae from other bacteria, and could be recommended as selective plating media (18).

Preenrichment sample size was an important factor for isolating *Salmonella* from cake mix samples; however, neither sample size was better, as *Salmonella* was isolated from one sample by only using 25 g and from the other sample only by using 100 g for preenrichment. It is likely the number of *Salmonella* cells in the cake mix were very low and unevenly distributed. These results indicate that both 25- and 100-g samples should be used to isolate *Salmonella* from dried food samples like cake mix.

In conclusion, *Salmonella* Typhimurium with a PFGE subtype indistinguishable of the outbreak isolate was recovered from two cake mix samples implicated in the outbreak. The best combination of conditions for isolating the outbreak strain was preenrichment of cake mix samples in universal preenrichment broth at 35°C for 24 h, followed by enrichment at 42°C for 24 h in TT broth. BGA, XLT4, XLD, HE, and BSA media were equally effective in isolating *Salmonella* from cake mix, and XLT4 and XLD sup-

pressed the growth of background microflora more effectively than the other media.

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