A Collaborative Study on a Method to Detect Salmonella in Food

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Fourteen laboratories with expertise in Salmonella detection in food joined in a collaborative study. The laboratories performed qualitative analyses of ground pork samples using the proposed detection method. Salmonella Typhimurium (hydrogen sulfide-producing strain) and Salmonella Senftenberg (hydrogen sulfide-nonproducing strain) were used for inoculation. Three levels of Salmonella contamination were used for the study (0, 1-10, and 11-100 cfu/25 g). We evaluated the presence of Salmonella in each sample and the serological O group. Unmarked samples delivered to the laboratories were accurately judged to be inoculated or not inoculated with Salmonella at a 99.8% (419/420) detection rate in this collaborative study. The proposed method is suitable as a standard method to detect Salmonella in food.

Key words: Collaborative study/Salmonella detection/Standard method.

Since 1993, Salmonella contamination in meat products such as ham, Vienna sausage, bacon, roast beef, and cured ham, has been determined by using a certain microbiological test described in a circular from the Ministry of Health, Labour and Welfare of Japan (MHLW) (Document file No.: Einyu No. 54, 1993. 3. 17). The test to detect Salmonella has been carried out with Enterobacteriaceae enrichment mannitol (EEM) broth as a pre-enrichment broth, with serenite brilliant green (SBG), serenite cystine (SC) or Hajna tetrahionate (TT) broth as a selective enrichment broth, and with deoxycholate hydrogen sulfide lactose (DHL) or mannitol lysine crystal violet brilliant green (MLCB) agar as the isolation agar plate. In addition, a pre-enrichment method using buffered peptone water (BPW) with an additive (cystein or FeSO4) has been introduced as a Salmonella detection method in liquid whole egg as described in a circular from MHLW (Document file No.: Seiei No. 1674, 1998.11.25). However, we need to develop a unified Salmonella detection method in food. The modified BPW enrichment methods have been used for investigations of pathogenic bacteria in food. A preliminary study based on the modified BPW enrichment method was carried out by three

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laboratories and the results were reported (Miyahara et al., 2009). A precollaborative study was carried out effectively by six laboratories. Finally, a multilaboratory collaborative study (involving 14 laboratories) was conducted to determine the minimum number of detectable cells using two different characterized Salmonella strains. We report on the results of the collaborative study and propose a standard method to detect Salmonella in food.

S. Typhimurium (hydrogen sulfide-producing strain, ATCC 14028, O1, 4, [5], 12: i, 1, 2) and S. Senftenberg (hydrogen sulfide-nonproducing strain, 18A-70 strain isolated from a patient, O1, 3, 19: g,s,t: −) were used for inoculation. Ground pork was purchased from a supermarket was used as the food sample. The meat was tested repeatedly for Salmonella contamination by the proposed detection method. Standard plating counts (SPC) of the meat were also carried out. Thirty sample bags that were sent out included 6 for control, 6 for the low-dose (L) group (1-10 cfu / 25 g) of S. Typhimurium, 6 for the high-dose (H) group (11-100 cfu / 25 g) of S. Typhimurum, 6 for low dose (L) S. Senftenberg and 6 for high dose (H) S. Senftenberg. The samples were randomly numbered. The test samples were sent to fourteen laboratories by parcel mail under chilled conditions. Twelve collaborative laboratories located in mainland of Japan received the test samples on the following day and other two collaborative laboratories located outside of mainland Japan received them after two days. The temperature conditions were recorded by a temperature logger (TL20, 3M, Hecho en, China) which was returned by mail after the end of the collaborative studies. The temperature logger was also used in the sample incubator during the test period. Inoculation numbers were calculated on the basis of 0.1-ml plating on 10 tryptic soy agar (Difco-Becton Dickinson, Sparks, USA) plates. The detection protocol is shown in Fig.1. All the reagents described as follows has been purchased and delivered ahead of time: BPW (MERCK), Rappaport-Vassiliadis (RV, OXOID, Hampshire, England), TT (OXOID), potassium iodide (KI), iodine (I₂), MLCB (NISSUI, Tokyo, Japan), brilliant green agar + 0.1% sulfapyridine (BGS, OXOID), triple sugar iron (TSI, NISSUI), lysine, iron, mortality (LIM, EIKEN, Tochigi, Japan), indole reagent solution (MERCK, Darmstadt, Germany), and multivalent O, O4 and O1, 3, 19 anti-sera (Denka, Gosen, Nihonl).

As shown in Fig.1, 225 ml of BPW was added to each stomacher bag and incubated for 22 ± 2 h at 35 ± 1°C. After incubation, 0.1 and 1.0 ml of the BPW culture were added to 10 ml of preheated RV and TT (with KI and I₂) broth, respectively. Both samples were incubated for 22 ± 2 h at 42 ± 0.5°C. After incubation, 10 μl of RV or TT broth was plated on MLCB and BGS (for detection of hydrogen sulfide-producing and -nonproducing Salmonella). Three typical Salmonella colonies were isolated from each selective agar plate (MLCB and BGS) and were inoculated in TSI and LIM. After the typical characteristics were confirmed to be those of Salmonella, the colonies on TSI were determined by the agglutination test using anti-serum O group. A typical colony of Salmonella on MLCB is black and on BGS, red-colored plate, is colorless. A typical colony of Salmonella grown on TSI and LIM is as follows: on TSI, the slant is red and the butt is yellow with a black mass; on LIM, the butt is brilliant violet and indole-reaction-negative. The final detection results for Salmonella were determined using the following criteria. If there was even one Salmonella colony from a sample, it was determined to be Salmonella-positive.

The mean SPC of ground pork was 4.5×10⁸ cfu/g. The ground pork was tested for Salmonella by the proposed method and was evaluated as Salmonella-free. The average low and high inoculation numbers of S. Typhimurium were 4.9 and 36.6 cfu/sample bag, respectively. The average low and high inoculation numbers of S. Senftenberg were 5.6 and 55.7 cfu/sample bag, respectively. The test temperature conditions were controlled properly by all the collaborators.

Salmonella colonies were detected using four selective agar plates (RV-MLCB, RV-BGS, TT-MLCB, and TT-BGS). In the control group, no Salmonella colony was detected (0/249). Colonies on the BGS plates were shown to be Salmonella at the detection

![FIG. 1. Method to detect Salmonella in food (Collaborative study)](image_url)
TABLE 1. Salmonella colony detection on MLCB and BGS

<table>
<thead>
<tr>
<th>Detection of Salmonella</th>
<th>Number of Salmonella colonies/Number of checked colonies suspected to be Salmonella (Detection rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV-MLCB</td>
</tr>
<tr>
<td>H₂S + S. Typhimurium L</td>
<td>249/249 (1.00)</td>
</tr>
<tr>
<td>H₂S + S. Typhimurium H</td>
<td>252/252 (1.00)</td>
</tr>
<tr>
<td>H₂S – S. Senftenberg L</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>H₂S – S. Senftenberg H</td>
<td>2/10 (0.2)</td>
</tr>
<tr>
<td>Total</td>
<td>503/512 (0.982)</td>
</tr>
</tbody>
</table>

TABLE 2. Results of the collaborative study on Salmonella detection in food

<table>
<thead>
<tr>
<th>Salmonella detection (O group)</th>
<th>Detection accuracy</th>
<th>Special comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>84/84</td>
</tr>
<tr>
<td>H₂S +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low inoculation no. with Salmonella Typhimurium</td>
<td>O4</td>
<td>83/84</td>
</tr>
<tr>
<td>High inoculation no. with Salmonella Typhimurium</td>
<td>O4</td>
<td>84/84</td>
</tr>
<tr>
<td>H₂S –</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low inoculation no. with Salmonella Senftenberg</td>
<td>O1,3,19</td>
<td>84/84</td>
</tr>
<tr>
<td>High inoculation no. with Salmonella Senftenberg</td>
<td>O1,3,19</td>
<td>84/84</td>
</tr>
</tbody>
</table>

rate of 0.990, (1001 + 983)/(1004 + 1000). On the other hand, colonies on the MLCB plates were shown to be Salmonella at the detection rate of 0.926, (503 + 484)/(512 + 554). Table 1 shows the colony detection results from the inoculated samples. The rate of detection of Salmonella colonies from RV broth ((503 + 1001)/(512 + 1004), 0.992) was higher than that from TT broth [(484 + 983)/(554 + 1000), 0.944]. More colonies from the samples inoculated with S. Typhimurium [(972 + 999)/(995 + 1006), 0.985] were detected compared with that from the samples inoculated with S. Senftenberg [(497 + 503)/(538 + 531), 0.935]. In the S. Typhimurium L group except for one sample, almost all the colonies detected were Salmonella. In the S. Typhimurium H group, almost all the colonies detected were Salmonella. In the MLCB plate of S. Senftenberg L and H group most colonies were pale violet. This is not a typical color for Salmonella, so such colored colonies were evaluated as Salmonella-negative. On the other hand, BGS colonies of S. Senftenberg were determined to be Salmonella.

As shown in Table 2, the final results were as follows. No Salmonella colony was detected in any of the control samples (84 bags). In the L group samples, S. Typhimurium was detected as the O4 group at a rate of 83/84. In one sample, Salmonella was not detected. In the H group samples, S. Typhimurium was detected at a rate of 84/84. In L samples, S. Senftenberg was detected as the O1, 3, 19 group at a rate of 84/84. In H samples, S. Senftenberg was detected at a rate of 84/84.

All the individual collaborators had already handled Salmonella in line with their work and the laboratories to which they belonged had already passed the criteria of external certification bodies. The official method of Salmonella detection in meat products (EEM broth enrichment, SC, SBG or Hajna TT selective enrichment and MLCB or DHL plating) has been used since 1993 and now the EEM enrichment method of Salmonella detection in meat products is used only in Japan. BPW and lactose broth are the standard enrichment broths for Salmonella detection in Europe and U.S.A, respectively. The BPW enrichment method (ISO 6579;2002 culture method for Salmonella) was adopted as the official first step for the analysis of freshly chilled and frozen poultry and dried egg products by comparison with the AOAC official method (Feldsine, et al., 2003). The release and transfer of selenium and selenite compounds must be
registered with MHLW. The proposed standard method was confirmed by this collaborative study. *Salmonella* under ten cells per stomacher bag (25 g food) could be detected by the method.

In Japan, most food-poisoning cases of *Salmonella* have been caused by hydrogen sulfide-producing strains except for a few cases (Kasahara, 1999). However, we need to monitor hydrogen sulfide-producing or nonproducing *Salmonella* contamination in food to protect our health. We already compared these two kinds of agar plates for *Salmonella* detection. One kind was for hydrogen sulfide-producing *Salmonella*, such as DHL, MLCB, xylose, lysine, and desoxycholate medium (XLD) and bismuth sulphite agar (BS), and the other was for hydrogen sulfide-producing and also for nonproducing *Salmonella*, such as CHROMagar *Salmonella* (CHS, CHROMagar, Paris), ES *Salmonella* agar II (ESII, Eiken, Tochigi), BGS and ChromID™ *Salmonella* agar. (SM2, Biomerieux, Lyon, France). These agar plates for *Salmonella* detection have been commonly used in the world. Methods used for comparison were the Misra method (Sakazaki et al., 1986) and sensitivity test by three inoculated *Salmonella* strains (S. Typhimurium, S. Senftenberg and S. Infantis) in food. The agar plates showed similar detection activity, but only BS could not show similar activity under the same conditions. Evaluation data of the agar plates have already been reported at the annual meeting (Taguchi et al., Japanese Society of Food Microbiology, 2007). Although we reported results of a collaborative study for a new method to detect *Salmonella* with two kinds of agar plates (MLCB and BGS), DHL and XLD for hydrogen sulfide-producing *Salmonella* and CHS, ESII and SM2 for hydrogen sulfide-producing and nonproducing *Salmonella* could be used as isolation agar plates.

We also compared the former method and this method for *Salmonella* detection in liquid whole egg samples. Egg samples (white, yolk and white) were inoculated with small amounts of *Salmonella* (under ten cells/25 g food). As results, not shown in this report, both kinds of enrichment induced the same level of detection. We could detect small amounts of *Salmonella* by the method studied not only in meat but also in egg samples.

We recommend our proposed method as a standard method of *Salmonella* detection in food (Fig.2). This method to detect *Salmonella* in food has the following features: (1) Small amounts of *Salmonella* (under 10 cells / 25 g) in food are detectable; and (2) Both hydrogen sulfide-producing or nonproducing *Salmonella* are detectable.

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