Salmonella Screening Procedure with Tests for 
β-Galactosidase and Flagellar Antigens

CLYDE R. WILSON, ADRIANO P. PADRON, AND W. B. DOCKSTADER
Division of Microbiology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D.C. 20204

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Differentiation of Salmonella from other gram-negative bacilli requires several biochemical and serological tests. A simplified 24-hr screening procedure has been devised which allows discarding of large numbers of isolates (picked from selective plating media) before they are subjected to this extensive testing. Cultures of gram-negative organisms isolated to triple sugar-iron slants during routine examination of products for Salmonella were tested for the presence of β-galactosidase and Salmonella flagellar antigens. β-Galactosidase-positive cultures which did not agglutinate in polyvalent flagellar antiserum were considered to be nonsalmonellae. Of 1,103 Salmonella cultures tested, none of the 61 different serotypes was missed by this procedure, whereas 673 (82.3%) of 818 nonsalmonellae were excluded from further testing. This screening procedure eliminates most nonsalmonellae and augments the proportion of cultures undergoing further biochemical and serological testing which will be confirmed as Salmonella.

Current identification procedures for Salmonella require considerable analytical time. Many suspect isolates must be regarded as Salmonella until definitely eliminated by a battery of biochemical and serological tests. There is a need for a screening procedure which results in the early elimination of nonsalmonellae cultures. This study describes such a screening procedure based upon hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) and reaction with polyvalent Salmonella flagellar antiserum.

The ONPG test is used to detect the presence of β-galactosidase which hydrolyzes ONPG to o-nitrophenol. This reaction is characterized by the formation of a yellow color in the medium. Among salmonellae, only the lactose-fermenting strains are known to carry this enzyme. The polyvalent flagellar test determines the presence or absence of antigens considered to be specific for Salmonella.

Johnston and Thatcher (6) have shown that growth of lactose-fermenting organisms may be inhibited by addition of phenethly β-D-galactopyranoside (PEG) to preenrichment media. The cleavage of this molecule results in the release of phenethyl alcohol which is toxic to most gram-negative organisms. This technique was impractical for routine separation of microbial contaminants because of the high cost of PEG. Furthermore, lactose-fermenting salmonellae would also be inhibited by hydrolysis of PEG. An approach is needed that would allow the retention of lactose-fermenting salmonellae which, even though known for many years (7), have become of increasing significance in foodborne illness (12). The procedure described here retains Salmonella, including those that ferment lactose, while allowing the elimination of a large percentage of nonsalmonellae.

MATERIALS AND METHODS

Except for the two ONPG broths, all media were commercially available in dehydrated form and were prepared according to the instructions of the manufacturer. The basal broth used for both ONPG media was 1.0% tryptone (Difco) with 0.5% NaCl and was sterilized at 121°C for 15 min. Tryptone was selected because it is nearly colorless in a 1.0% solution; darker (yellow) media may obscure weak positive reactions. The ONPG solution was prepared by dissolving 1.0 g of ONPG (Calbiochem) in 167.0 ml of 0.1 Μ KH2PO4 solution (pH 7.4) and was sterilized by filtration.

The 24-hr ONPG test medium, modified from the formula of Cowan and Steel (2), was prepared by adding 25.0 ml of ONPG solution to 75.0 ml of the cooled basal broth. Two-milliliter amounts of the finished medium were dispensed aseptically into sterile tubes (13 by 100 mm). These tubes were incubated for 24 hr at 37°C, checked for contamination, and refrigerated at 4°C until used.

The medium for the rapid ONPG test, modified from the work of Pickett and Goodman (11), was prepared by adding 9.0 ml of the ONPG solution to 91.0 ml of cooled basal broth containing 1.0 mg of sodium
azide per ml. The complete medium was stored in bulk at 4°C; at the time of use, 0.3-ml amounts were dispensed into tubes (10 by 75 mm).

Final concentrations of ONPG per milliliter in the 24-hr and rapid-test broths were 1.5 and 0.5 mg, respectively. On a per test basis, the quantity of ONPG used was 3.0 mg for the 24-hr test and 0.15 mg for the rapid test. It is essential that the pH of both ONPG media be 7.2 to 7.4 for proper manifestation of the color reaction (9).

All cultures used in this study had been isolated from food or feed samples by conventional methods as suspect salmonellae. Therefore, they had passed through a selective broth and had subsequently been picked from a selective agar. Each culture was transferred onto a triple sugar-iron (TSI)-agar slant (3 ml in a tube, 13 by 100 mm) and incubated for 18 to 24 hr at 37°C to simulate a fresh isolate. Growth from the TSI-agar slant was used to inoculate 1.0% tryptone broth (10.0 ml in a 16 by 150-mm tube), phenol red broth base with 1.0% lactose (3.0 ml in a 13- by 100-mm tube with a Durham tube), and one of each ONPG broth.

All media except the rapid-test ONPG broth were inoculated with sufficient cells to initiate growth and incubated in a warm air incubator at 37°C. The ONPG rapid-test medium requires a much heavier inoculum since the reaction is dependent upon existing cells. A 3-mm loopful of culture taken from a TSI slant proved to be a satisfactory inoculum when suspended in 0.3 ml of rapid-test medium. These tubes were placed in a water bath at 37°C, read at intervals, and left in the water bath for 24 hr.

Antigen for the flagellar test was prepared by inactivating the 24-hr tryptone broth culture with 5.0 ml of physiological saline containing 0.6% formaldehyde. Polyvalent flagellar antiserum (Salmonella “H” Antiserum Poly, Difco) was diluted so that 0.02 ml of antiserum in 1.0 ml of antigen gave the manufacturer’s recommended titer of 1:100 (3). Antigen was added to the antiserum in a tube (10 by 75 mm), and the tube was incubated in a water bath at 50°C for 1 hr and checked for agglutination (4).

RESULTS

A comparison of lactose utilization (lactose broth) and the presence of β-galactosidase (ONPG broth) in 818 nonsalmonellae cultures is given in Table 1. These data indicate that β-galactosidase was present in 714 of the 818 cultures tested. The reactions in lactose broth, on the other hand, indicated that β-galactosidase (positive lactose fermentation) was present in 508 cultures of the 818 tested. In other words, 310 cultures would have been subjected to the additional battery of biochemical and serological tests required for Salmonella identification if lactose broth fermentation alone had been used as a screening procedure. If the ONPG test alone had been used as a screening procedure, only 104 cultures would have received further testing. This test alone, of course, would not detect the occasional lactose-positive Salmonella.

Table 2 presents data on the correlation between the presence of β-galactosidase as determined by the ONPG test and Salmonella “H” antiserum with cultures of gram-negative bacilli. Of 1,103 known cultures of salmonellae, only three yielded positive ONPG test results. Within this same group of 1,103 cultures, 11 cultures showed negative reactions for Salmonella by means of the polyvalent “H” agglutination test. This comparison indicates that fewer false-negative reactions for Salmonella were obtained by the ONPG test alone than by the polyvalent “H” antigen test alone.

In reviewing the results obtained by the two test procedures when they were applied to nonsalmonellae cultures, a different picture was obtained. The ONPG test results revealed that 12.7% of the 818 test cultures yielded reactions that are expected of salmonellae (negative ONPG), whereas only 7.5% of the total cultures yielded serological test reactions typical of Salmonella (agglutination in polyvalent “H” antiserum). This comparison indicates that the ONPG test alone is not as reliable as the polyvalent “H” test alone for screening out nonsalmonellae cultures.

When the combined results of both tests are reviewed, it is apparent that four reaction patterns are possible. These reaction patterns are listed in Table 3 as are the recommended actions to be taken when cultures display these reaction patterns.

Pattern 1 (ONPG-positive, polyvalent “H”-negative): Although it is possible for lactose-fermenting salmonellae with damaged flagella to give this pattern, none was encountered.

Pattern 2 (ONPG-negative, polyvalent “H”-positive): Typical salmonellae give this pattern, as do nonsalmonellae which are β-galactosidase-positive.

| Reaction pattern | Total no. | Per cent |
|------------------|-----------|----------|
| ONPG | Lactose broth | |
| + | + | 508 | 62.1 |
| + | - | 206 | 25.1 |
| - | - | 104 | 12.8 |
| - | + | 0 | 0 |

*All 818 cultures were demonstrated not to be members of the genus Salmonella by biochemical and serological tests.*
negative and possess cross-reacting flagellar antigens.

Pattern 3 (ONPG-positive, polyvalent "H"-positive): Lactose-fermenting salmonellae with normal flagellar response give this pattern, as do nonsalmonellae possessing β-galactosidase and cross-reacting flagellar antigens.

Pattern 4 (ONPG-negative, polyvalent "H"-negative): S. pullorum, S. gallinarum, and non-lactose-fermenting salmonellae with damaged flagellar antigens, or antigens for which the antiserum has no antibodies, give this pattern, as do nonsalmonellae possessing neither β-galactosidase nor cross-reacting flagellar antigens.

**DISCUSSION**

Using only the ONPG test for excluding nonsalmonellae would result in discarding lactose-fermenting salmonellae. LaPage and Jayaraman (8), in their testing of 1,075 stock cultures of Salmonella, reported nine serotypes as ONPG-positive, including four strains with irregular biochemical properties which had been characterized as non-lactose fermentors. Because of increasing concern over these organisms, a second test was included to retain them. Many tests were considered and tried before the decision was made to use polyvalent Salmonella flagellar antiserum. Flagellar serology proved to be highly specific, although not to the extent reported by Hajna and Damon (5). The antiserum they used, however, was obtained from a noncommercial source and was not identical to that used in this study. Organisms from culture collections of salmonellae and other Enterobacteriaceae were not subjected to study since this work has been adequately done. As Lubin and Ewing (10) pointed out, results from work with stock cultures from culture collections could not be extrapolated to apply to prevailing natural flora.

Using only flagellar serology for screening involves the possibility of overlooking some salmonellae. The only two serotypes listed as nonmotile by the Kauffmann-White schema of 1964 are S. pullorum and S. gallinarum. However, from materials subjected to high heat and other severe processing treatments, this laboratory has isolated numerous salmonellae which were damaged and no longer exhibited flagellar agglutination. Biochemical characterization and somatic serology defined these cultures as Salmonella. However, many remained nontypable because of their lack of "H" antigens.

Another consideration is the absence of certain factors from the antisera now available. Testing with polyvalent flagellar antiserum alone would result in discarding S. pullorum, S. gallinarum, all salmonellae with unreactive flagellar antigens, and all salmonellae with flagellar antigens for which the antiserum contained no antibodies.

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**Table 2. Correlation of the presence of β-galactosidase and Salmonella H antigens in cultures of gram-negative bacilli**

| Type of culture | No. of cultures | ONPG reaction | Polyvalent H agglutination |
|-----------------|----------------|---------------|----------------------------|
| Salmonella      | 1,103          | 3 (0.3)<sup>a</sup> | 1,100 (99.7)               |
| Nonsalmonellae  | 818            | 714 (87.3)    | 1,092 (99.0)               |
|                 |                |               | 11<sup>c</sup> (1.0)       |

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**Table 3. Reaction patterns of Salmonella and other gram-negative bacilli**

| Type of culture | No. of cultures | Reaction pattern                                      |
|-----------------|-----------------|-------------------------------------------------------|
|                 |                 | (1) ONPG-positive, poly H<sup>-</sup>-negative        |
|                 |                 | (2) ONPG-negative, poly H<sup>+</sup>-positive        |
|                 |                 | (3) ONPG-positive, poly H<sup>-</sup>-positive        |
|                 |                 | (4) ONPG-negative, poly H<sup>+</sup>-negative        |
| Nonsalmonellae  | 818             | 673 (82.3)<sup>c</sup>                                |
| Salmonella      | 1,103           | 0                                                     |
|                 |                 | 20 (2.4)                                              |
|                 |                 | 41 (5.0)                                              |
|                 |                 | 84 (10.3)                                             |
|                 |                 | 1,089 (98.7)                                          |
|                 |                 | 3 (0.3)                                               |
|                 |                 | 11 (1.0)                                              |
However, the data indicate that using the ONPG test in conjunction with the “H” agglutination test minimizes the probability of discarding atypical salmonellae.

If desired, results of the flagellar test may be obtained the same day as results of the rapid ONPG test by inoculating a tube of Brain Heart Infusion (BHI) broth or “H” broth (5) before the rapid ONPG test is begun. Our results indicate that a 6-hr BHI or “H” broth culture regularly produces sufficient antigen for the flagellar test.

Attempts were made to use a water bath at 50°C for the rapid ONPG test, but results were unfavorable. Some weak positive reactions were inhibited, and the color of all tubes faded considerably when left in the water bath at 50°C overnight, as might occur if examination were begun late in the day. Little or no fading was noticed in tubes incubated in the water bath at 37°C for periods as long as 72 hr. Most positive reactions occurred within 2 hr, and, according to Bülow (1), a final reading may be made after 3 hr.

Of the 1,103 salmonellae cultures tested, all but 48 were identified to serotype. These 48 cultures were definitely classified as being *Salmonella* species by biochemical tests and somatic serology and were retained by the system even though many would not respond normally to standard serotyping techniques. Though the 61 serotypes encountered in this study comprise less than 5.0% of the known serotypes, these appear to be representative of serotypes most frequently isolated from nonhuman sources in the United States at the present time. The Annual Summary of the National Communicable Disease Center for 1968 (13) lists 10 serotypes which accounted for 56.0% of the nonhuman isolates, with the tenth ranked organism having a frequency of only 3.0%. Those 10 serotypes accounted for approximately 40.0% of the salmonellae encountered in this study. These data indicate that our laboratory encounters a wider range of salmonellae serotypes. However, even uncommon serotypes tested in this system gave characteristic patterns.

Current methodology makes no provision for discarding a TSI slant by visual examination alone. Since *Salmonella* reactions on TSI can be acid-butt/alkaline-slant with or without H₂S, or acid-butt/acid-slant with or without H₂S, most isolates on TSI would have to be regarded as being suspect *Salmonella*. By applying the ONPG and “H” agglutination tests immediately after obtaining an 18- to 24-hr TSI slant, most of the nonsalmonellae can be discarded. The criterion for exclusion by these tests is definitive and does not rely on judgement alone. This would be helpful to workers who had not yet gained expertise with *Salmonella*. Cultures giving the typical *Salmonella* pattern could be designated “presumptive positive” and given priority for subsequent biochemical and serological confirmation. Although 24 hr may be added to the analytical time for those isolates which are *Salmonella*, this would be of little consequence in the examination of foods or raw ingredients, since these materials should not be used or shipped until determined to be free from *Salmonella*. However, in situations in which the isolate is not a *Salmonella*, the material would be available for use sooner than is now possible. This set of tests lends itself to manufacturing quality control programs and to situations where many batches of raw materials, such as imported ingredients, must be screened for the presence of *Salmonella*. This procedure replaces none of the currently accepted methods used in testing for *Salmonella*, but it does reduce the probability of running exhaustive biochemical characterizations on suspect cultures which resemble *Salmonella*.

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