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Enumeration of Salmonellae by Most-Probable-Number Using the *Salmonella* 1-2 Test

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ABSTRACT

A rapid *Salmonella* identification test was substituted for the selective plating and identification steps in the standard cultural most-probable-number procedure. The modified procedure was evaluated using a mixed culture of four salmonellae species inoculated into ground beef. The rapid test kit was inoculated from both the pre-enrichment and selective enrichment steps in the standard procedure. There were fewer false negatives when the test kits were inoculated from selective vs. non-selective broths. However, there were no false negatives when the kit was inoculated from Rappaport-Vassiliadis' broth, after pre-enrichment in lactose broth. Use of the test kit in the MPN procedure produced acceptable results in 48 to 72 h, compared with 96 to 120 h for the standard procedure.

Although direct plating for the enumeration of salmonellae has been used experimentally *(8)*, the method of choice for most food products is the most-probable-number (MPN) technique. The conventional method for enumerating salmonellae by MPN is a tedious and time consuming process *(14)*. The method is an extension of the standard method for the isolation of salmonellae *(2)*, and involves multiple tube non-selective enrichment, selective enrichment, selective plating, and identification. This method requires a considerable amount of materials and labor, and can take as long as 120 h to complete.

Recently, several rapid tests have been developed to shorten the time required to isolate and identify salmonellae in foods. These have included hydrophobic membrane filtration *(10)*, immunological assays *(13)*, as well as DNA hybridization *(9,11)*. Several of these rapid tests, including an immuno-precipitin method have received AOAC approval *(1)*. Since many of these tests require pre-enrichment in lactose broth, it seemed logical that they could be used in an MPN procedure at that point in the procedure.

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[2]*Mention of trade name, proprietary products of specific equipment does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.*

The *Salmonella* 1-2 test is designed as a rapid screening test for salmonellae in foods. The test consists of a selective enrichment chamber separated from a non-selective motility chamber. The selective enrichment chamber is inoculated, and a plug removed to allow the sample to move into the motility chamber. If present, the salmonellae react with flagellar antigens in the motility chamber and form an immuno-precipitin band. Because the identification is based on flagellar antigens, the kit will not detect non-motile salmonellae *(1)*. The manufacturer recommends selective-enrichment in tetrathionate broth for raw meats and pre-enrichment in lactose broth followed by selective enrichment in tetrathionate broth for processed meats prior to inoculation *(3)*.

The objective of this study was to simplify the MPN procedure by using the *Salmonella* 1-2 test kit and to compare the modified MPN to the conventional MPN procedure. The modified procedure was also evaluated using injured salmonellae.

MATERIALS AND METHODS

Test Cultures

Cultures of *Salmonella typhimurium* (ATCC 14028), *Salmonella enteritidis* (ATCC 13076), *Salmonella arizonae* (ATCC 13314), and *Salmonella pullorum* (ATCC 19945) were grown and maintained on tryptic soy agar (TSA, Difco) at 32°C. The cultures were grown for 18 h at 32°C and harvested by centrifugation (3000G x 10 min 4°C). The pellets were resuspended in 9 ml Butterfield's phosphate buffer *(12)*. A mixed culture was prepared by mixing 2.5 ml of each of the washed cultures in a sterile test tube.

Mixed culture

The mixed culture was serially diluted in phosphate buffer to a concentration of approximately 10^3 colony forming units per ml (cfu/ml). The culture was plated using the pour plate technique *(7)* and incubated at 37°C for 48 h to give an initial control count. The remaining culture was inoculated into 9 ml lactose broth (Difco) tubes, using a three tube MPN series, as outlined by Kent et al. *(14)*. After 24 h at 37°C, 0.1 ml from each positive (turbid) tube was transferred individually to 9 ml

of selenite-cystine broth (SC, Difco), 9 ml of tetrathionate broth with brilliant green (0.01g/l) (TT, Difco) *(2)*, or the *Salmonella* 1-2 (TM) test (Biocontrol, Bothell, WA). Tubes which did not show turbidity were considered to be negative. The SC and TT broths were incubated at 37°C for 24 h, then streaked for isolation on Brilliant Green Agar with sulfadiazine (BGS, BBL). The BGS plates were incubated at 37°C for 24 h. Growth on selective agar was considered positive for the respective tube and dilution. The *Salmonella* 1-2 test was inoculated according to the manufacturer's instructions. One drop of iodine reagent was added to the inoculation chamber, and the test unit was gently agitated to achieve a uniform mixture. One drop of the antibody preparation was added to the gel void, making sure that no bubbles were present in the void after addition. The inoculation chamber plug was removed, and 0.1 ml from each turbid lactose tube was added to the chamber. Tubes which lacked turbidity were considered to be negative. The test units were incubated with the inoculation chamber in the vertical position at 37°C for 18-24 h, and then examined for the presence of an immuno-precipitin band, indicating a positive result. The most-probable-number per ml (MPN/ml) was calculated using standard three tube MPN table *(4)*.

Inoculated ground beef

Ground beef was obtained from the abattoir at the U.S. Meat Animal Research Center, separated into 100 g quantities, and frozen in sterile bags until needed. Prior to inoculation, the meat was thawed overnight at ambient temperature (ca. 23°C) to increase the populations of bacteria. Total plate counts and *Enterobacteriaceae* counts were performed on the meat with TSA and Violet Red Bile Glucose agar (VRBG, Oxoid), respectively, using the pour plate technique. The plates were incubated at 32°C for 48 and 24 h, respectively. The mixed culture was prepared as outlined above, diluted in phosphate buffer to give approximately 10^4 cfu/ml, and pour plated with TSA as a control. One ml of this dilution was added to 11 g of ground beef and mixed manually. The inoculated meat was stomached for 2 min in 99 ml lactose broth. Ten ml of the stomached sample was transferred to each of 3 sterile tubes. The stomached sample was then serially diluted in phosphate buffer, and each dilution was inoculated into 3, 9 ml lactose broth tubes (1 ml dilution/tube). Lactose broth tubes were incubated at 37°C for 24 h, and 1 ml from each positive tube was transferred to SC, TT, Rappaport-Vassiliadis' broth (R-V, Oxoid) *(6)* and the 1-2 test. Tubes which did not show turbidity were considered negative. The 1-2 test was performed as described above. The selective broths were incubated for 24 h at 37°C, and then streaked for isolation on Bismuth Sulfite agar (BSA, Difco). The 1-2 test was also inoculated from the selective broths. The selective plates were incubated at 37°C for 24 h, and colonies showing typical *salmonella*-like morphology and reaction were transferred to triple sugar iron agar (TSI, Difco) and lysine iron agar (LIA, Difco) slants for confirmation.

Injured bacteria

One ml of the mixed culture was added to 9 ml of 2% acetic acid (vol/vol) and allowed to incubate at room temperature for 2 min. Two ml was then transferred to 18 ml phosphate buffer containing sufficient 0.1 N NaOH to neutralize the acid. Plate counts were performed using TSA and VRBG, and the percent injury was calculated by comparing the two counts. The injured culture was serially diluted in phosphate buffer and inoculated into ground beef. The experiment was performed as outlined above, although only SC and TT were used as selective enrichment broths. Lactose and tetrathionate broths were used to inoculate the 1-2 tests.

RESULTS AND DISCUSSION

As expected, the results of the two MPN methods were identical with the mixed culture in broth. The initial population as determined by plate count was 2.64 $\log_{10}$ colony forming units/ml. Both the standard and 1-2 test MPN procedures produced counts of 2.24 $\log_{10}$ most-probable-number/ml. The differences in count between the direct plate count and the MPN method are due to the inherent sensitivities in both methods. Although the MPN results are lower than the direct counts, the direct counts fall within the 95% confidence intervals for the MPN.

The total aerobic and *Enterobacteriaceae* counts for the ground beef averaged $\log_{10}$ 6.91 and 6.33, respectively. There were fewer false negatives when the 1-2 kits were inoculated directly from selenite-cystine broth than form tetrathionate and lactose broths, although there were no false negatives when the kits were inoculated from R-V broth (Table 1). When the test kits were inoculated from SC broth, there were difficulties in interpreting the test results. The SC broth clouded the agarose in the test kit, making it difficult to see the precipitin band. The isolates from all of the false negatives tubes were identified as non-motile salmonellae, i.e., *S. pullorum*. The results may indicate that TT is more toxic to the other species used in this experiment. However, the 1-2 test will only detect motile species, and this undoubtably accounts for some of the false negatives.

Tongpim et al. *(15)* reported generally higher MPN counts of both naturally and artificially contaminated samples with Rapapport-Vassiliadis' medium than with Muller-Kauffmann tetrathionate broth. R-V medium is also more consistent in detecting salmonellae in the *salmonella* isolation procedure and has been recommended as a replacement for tetrathionate broth *(5)*. R-V medium is apparently a better inoculum source for the 1-2 test kits than tetrathionate broth.

The acid injured salmonellae were more difficult to detect using the modified method (Table 2). The average percent injury (TSA count - VRBG count)/TSA count) x 100) on the acid injured inoculum was 93%. There were 6 false negatives when the 1-2 tests were inoculated from lactose broth, but only 3 when the tests were inoculated from TT. The results after pre-enrichment (i.e., inoculated from TT) were similar to those for the non-injured salmonellae. The isolates from the false negative lactose tubes included all four strains of the mixed culture. However, only *S. pullorum* was isolated from the false negative selective enrichment tubes. Some of the false negatives may be caused by a lower selectivity in the 1-2 inoculation chamber, i.e., small inoculation volume. However, the selective enrichment step is necessary after the lactose pre-enrichment if there is a possibility of injured bacteria in the initial sample.

TABLE 1. *Comparison of* Salmonella *1-2 MPN with standard cultural MPN procedure: Inoculated ground beef summary.*

1-2 Inoculum[1]	Expt. Number	MPN 1-2[2]	Sequence STD		Number of (STD(+) 1-2(-)[3]	Source of (+) tubes[4]
Lactose	1	3-3-1	3-3-2		1	TT
	2	3-3-2	3-3-2		0	
	3	3-3-1	3-3-1		0	
	4	2-2-0	3-3-0		2	SC
				Total	3	
TT	1	3-3-2	3-3-2		0	
	2	3-2-0	3-3-2		3	SC
				Total	3	
SC	1	3-2-0	3-2-0		0	
	2	3-2-0	3-3-0		1	SC
				Total	1	
R-V	1	3-3-1	3-3-1		0	NA
	2	3-3-0	3-3-0		0	
	3	3-2-0	3-2-0		0	
	4	3-3-0	3-3-0		0	
				Total	0	

[1]Source of inoculum for 1-2 test; TT = tetrathionate, SC = selenite cystine, R-V = Rapapport-Vassiliadis broths.
[2]Most-probable number sequence produced by 1-2 MPN or standard cultural (STD) MPN procedure. Number of positive tubes per dilution, based on 3 tubes/dilution.
[3]Number of tubes positive by STD which were negative by 1-2 MPN.
[4]Selective broth from which additional STD positive tubes were isolated from; NA = not applicable.

TABLE 2. *Comparison of* Salmonella *1-2 MPN with standard cultural MPN procedure: Acid injured bacteria.*

1-2 Inoculum[1]	Expt. Number	MPN 1-2[2]	Sequence STD		Number of STD(+) 1-2(-)[3]	Source of (+) tubes[4]
Lactose	1	3-3-0	3-3-0		0	
	2	2-1-0	3-3-2		5	SC
	3	3-2-2	3-3-2		1	SC
				Total	6	
TT	1	3-3-0	3-3-0		0	
	2	3-2-0	3-3-2		3	SC
	3	3-3-2	3-3-2		0	
				Total	3	

[1]Source of inoculum for 1-2 test; TT = tetrathionate, SC = selenite cystine, R-V = Rapapport-Vassiliadis broths.
[2]Most-probable number sequence produced by 1-2 MPN or standard cultural (STD) MPN procedure. See footnote 2, page 12.
[3]Number of tubes positive by STD which were negative by 1-2 MPN.
[4]Selective broth from which additional STD positive tubes were isolated from; NA = not applicable.

The standard cultural MPN method involves many transfers and requires 96 to 120 h to perform. When the *Salmonella* 1-2 test is substituted for selective plating and identification, the procedure can be shortened to 72 h or less. In addition, the modified procedure is less complicated to perform. Subsequent use of this method in our laboratory has proven it to be a reliable and efficient method of enumerating salmonellae in diverse samples.

CONCLUSIONS

A most-probable-number procedure is usually performed on samples which have tested positive for salmonellae. The modified procedure is an acceptable alternative to the standard cultural method when it is known that the strain or strains of *salmonella* present in the sample are motile. The best correlations were obtained when the 1-2 kits were inoculated from Rappaport-Vassiliadis' broth, after preenrichment in lactose broth. A five tube MPN would be more precise and have narrower confidence intervals than a 3 tube MPN *(4)*, and the modified procedure presented here could readily be adapted to a five tube procedure. It is likely that some of the other rapid *salmonella* tests could be substituted in this procedure with similar results.

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