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Research Paper

Development and Validation of a Cultural Method for the Detection and Isolation of *Salmonella* in Cloves

GUODONG ZHANG,* LAILA ALI, VIKAS GILL, APARNA TATAVARTHY, XIAOHONG DENG, LIJUN HU, ERIC W. BROWN, AND THOMAS S. HAMMACK

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5001 Campus Drive, College Park, Maryland 20740, USA

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ABSTRACT

Detection of *Salmonella* in some spices, such as cloves, remains a challenge due to their inherent antimicrobial properties. The purpose of this study was to develop an effective detection method for *Salmonella* from spices using cloves as a model. Two clove varieties, Ceylon and Madagascar, were used in the study. Cloves were inoculated with *Salmonella enterica* subsp. *enterica* serotypes Montevideo, Typhimurium, or Weltevreden at about 1, 3, or 6 log CFU/25 g. Two test portion sizes, 10 and 25 g, were compared. After adding Trypticase soy broth (TSB) to the weighed cloves for preenrichment, three preenrichment methods were compared: cloves were left in the TSB for 24 h during preenrichment (PreE1), or the cloves-TSB mixture was shaken vigorously for 30 s (PreE2) or 60 s (PreE3), and the decanted material was transferred to a new bag for 24 h of preenrichment. The rest of the procedures were carried out according to the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (BAM). At the low inoculation level (<1 log CFU/25 g), the detection rate was low across the three preenrichment methods, with the highest for PreE3 and lowest for PreE1. At the medium and high inoculation levels (3 and 6 log CFU/25 g), all samples from PreE2 and PreE3 were positive for *Salmonella,* whereas PreE1 produced only 12 positive samples from the 48 samples at the medium inoculation level and 38 positive samples from the 48 samples at the high inoculation level. Therefore, PreE3 with 25 g of cloves per sample was more effective than the other two tested methods. This newly designed method was then validated by comparing with the BAM method in six trials, with each trial consisting of 40 test samples. The results showed that PreE3 detected *Salmonella* from 88 of 120 inoculated test samples compared with only 31 positive from 120 test samples with the BAM method. Thus, our newly designed method PreE3 was more sensitive and easier to operate than the current BAM method for detection of *Salmonella* in cloves.

Key words: Clove; Microbial detection; Preenrichment; *Salmonella*; Spice; U.S. Food and Drug Administration *Bacteriological Analytical Manual*

Spices have been consumed in every culture since ancient times. They add flavor and taste to foods, provide nutrition, and can have medicinal functions. In the last couple of decades, we have been strongly encouraged to reduce fats, salt, and sugar in our diet because of their negative health effects when used in excess. Spices play a more important role in preparing tasty and nutritional foods, complementing the other unhealthy but palatable ingredients. According the U.S. Department of Agriculture *(2, 15),* spice consumption has been on the rise in recent years. In June 2016, the U.S. Food and Drug Administration (FDA) *(19)* released draft guidance for sodium reduction targets in processed foods, which may further boost the usage of spices.

FDA recall and outbreak data clearly indicate that spices and seasonings are vehicles for salmonellosis. *Salmonella* contamination of spices was the cause of 95% of the U.S. food recalls associated with spices from 1969 to 2003 *(22)*. The only exception was a recall of bay leaves contaminated with *Listeria monocytogenes*. Three large-scale salmonellosis outbreaks that occurred between 2007 and 2010 in the United States were attributed to the consumption of *Salmonella*-contaminated spices or seasonings *(3, 21)*. By the end of its second year, in September 2011, the FDA Reportable Food Registry had recorded spices and seasonings as leading nearly all human food categories in total number of primary entries and the number of primary entries associated with *Salmonella (16)*. Reports from Europe have also linked many *Salmonella* infection outbreaks to contaminated spices *(8–10, 12, 13)*.

An extensive survey on the microbiological status of dried spices and herbs was conducted in the United Kingdom during 2004 *(14)*. Samples tested included pepper (red, black, white, and green), paprika, chili, aniseed, allspice, cinnamon, cumin, fennel, garam masala, ginger, mace, mustard, nutmeg, saffron, turmeric, basil, bay, coriander, oregano, parsley, rosemary, sage, thyme, and tarragon. *Salmonella* was detected in 1.5% of production batches and 1.1% of retail samples of dried spices and herbs.

* Author for correspondence. Tel: 240-402-2943; Fax: 301-436-1055; E-mail: guodong.zhang@fda.hhs.gov.

Salmonella enterica Senftenberg, Montevideo, and Typhimurium were the most frequently detected serovars. Ninety percent of samples examined were recorded as being ready to eat, but the majority of them did not appear to have had any processing treatments *(14)*. Hara-Kudo et al. *(7)* assayed 259 samples of 40 types of spices in Japan for *Salmonella* prevalence and found *Salmonella* serotypes Weltevreden and Senftenberg in black pepper and red pepper samples, respectively, but at very low levels of contamination (1 CFU/25 g). Banerjee and Sarkar *(1)* tested 154 samples of 27 types of spices from retail shops in India to determine spice microbial quality. Most of the tested samples were contaminated with microorganisms: molds were detected in 97%, *Bacillus cereus* and *Enterobacteriaceae* in 85%, *Staphylococcus aureus* in 59%, and *Salmonella* and *Shigella* in 2.6% of the samples. In the United States, a survey from 2007 to 2009 of *Salmonella* in imported spices prior to entry into the country revealed that 6.6% of the spices contained *Salmonella (20)*. Even among spices treated with pathogen reduction processes, 3% were contaminated, and *Salmonella* Weltevreden, Newport, Mbandaka, Agona, Bareilly, Montevideo, and Typhimurium were the most frequently found serotypes. A literature review *(26)* revealed that many *Salmonella* serovars were detected in a variety of spices and herbs. Overall, 0 to 8.4% of the samples were positive for *Salmonella,* albeit contamination rates were at the lower end of this range in most studies.

Many spices and seasonings are consumed in almost every household and in every restaurant every day. Considering that people habitually add raw spices and seasonings to salads, freshly cooked dishes, and many other types of ready-to-eat foods, spices are regarded as an important reservoir of *Salmonella (4)*. Because the United States has been increasing its importation of these products from regions where less than ideal sanitation conditions prevail, *Salmonella* infection outbreaks linked to contaminated spices or seasonings may continue to occur and increase.

The current FDA standard detection and isolation method for *Salmonella* in spices is that in the *Bacteriological Analytical Manual* (BAM) *(18)*. However, none of the current methods effectively neutralize the antimicrobial substances in spices such as allspice, cinnamon, and cloves. The current strategy used in the BAM *Salmonella* culture method for these matrices is to dilute the antimicrobial substances beyond their toxic concentrations. Allspice, cinnamon, and oregano are preenriched at a sample/broth ratio of 1:100 and cloves are preenriched at 1:1,000. This requirement poses a great challenge for following the BAM sampling plan, which mandates analysis of 20 25-g samples *(18)*.

The Pathatrix Auto System (Thermo Fisher Scientific, Waltham, MA), including an immunomagnetic separation step, has been proposed as a way to improve the detection of *Salmonella* in foods *(11, 24)*. In this study, we evaluated the effectiveness of this method for the detection of *Salmonella* in cloves. The main objective of this project was to develop an effective detection and isolation method for *Salmonella* from spices with inherent antimicrobial properties, using cloves as a model.

MATERIALS AND METHODS

Bacterial strains. Three *Salmonella* isolates were used in this study: *Salmonella* Montevideo (SAL1436), *Salmonella* Typhimurium (SAL3145H), and *Salmonella* Weltevreden (SAL2867). All isolates were obtained from FDA stock cultures (Division of Microbiology, Center for Food Safety and Applied Nutrition).

Cloves. The two types of cloves (flower buds) used for this study, from Ceylon and Madagascar, were purchased from an online wholesale spice retailer in the United States.

APCs. Total aerobic plate counts (APCs) for the clove samples were determined by adding 450 ml of buffered peptone water (Difco, BD, Sparks, MD) to 50 g of uninoculated cloves in a sterile filter bag (Whirl-Pak, Fisher Scientific, Pittsburgh, PA) and shaking vigorously for 1 min. Rinsate was serially diluted, and 100 μl of each dilution was spread plated on plate count agar plates (Difco, BD) and incubated at 35 ± 2°C for 48 h before reading.

***Salmonella* inoculum preparation.** *Salmonella* strains were grown in brain heart infusion (BHI) broth (Difco, BD) by transferring a single colony from a tryptic soy agar (TSA; Difco, BD) plate into 10 ml of BHI broth and incubating for 24 ± 2 h at 35 ± 2°C. *Salmonella* cells were then pelleted by centrifuging 10 ml of the overnight culture at 3,090 × *g* for 11 min. The harvested cells were then resuspended in 10 ml of Butterfield phosphate buffered water (BPBW; Hardy Diagnostics, Santa Maria, CA) and centrifuged again under the same conditions for 11 min. The wash step was repeated one more time, and the pellet was resuspended in 2.5 ml of BPBW and 2.5 ml of sterile 5% nonfat instant milk powder (from a local grocery store) solution. The entire 5-ml mixture was vortexed vigorously and poured into a 2-oz (60-ml) glass bottle (Boston round, Discount Vials, Madison, WI). The glass bottle was then rotated briefly on rollers with a shell bath (Vertis Freezemobile Dual FM, SP Scientific, Warminster, PA) temperature of –80°C following the manufacturer's instructions until the mixture was frozen. The bottle with the coated cell suspension was freeze dried for 18 h in a pilot lyophilizer (Genesis, SP Scientific). For the freeze drying, the shelf was initially cooled at –40°C for 80 min with the condenser temperature at –45°C. In the primary drying cycle, the shelf was heated to –20°C when the full vacuum was produced. In the secondary drying stage, the temperature of the shelf was set at 27°C with the postheat setting at 30°C for 240 min. The entire dried cell suspension was then aseptically scraped off the bottle and diluted in instant nonfat dry milk powder (Barry Farm Foods, Wapakoneta, Ohio) to achieve the desired concentration for inoculation. One gram of the final dilution of the milk powder inoculant was serially diluted and plated to determine *Salmonella* levels.

Dry inoculation. Cloves were inoculated with each milk powder *Salmonella* serotype inoculant. The inoculated cloves were mixed well aseptically and stored in a Ziploc bag in a cool dry environment for a minimum of 2 weeks before microbiological assays were conducted.

New detection and isolation method for *Salmonella* from cloves. A total of six trials were conducted with each of the two clove types tested against the three *Salmonella* serotypes. In each trial, three preenrichment methods were compared at three inoculation levels of about 1, 3, and 6 log CFU/25 g (Table 1) and two sample sizes of 10 and 25 g, with four replicates for each experiment. A total of 432 test portions were analyzed for three

TABLE 1. *Inoculation levels of* Salmonella *in cloves for development of a new detection method*

	Inoculation level (log CFU/25 g)					
	Ceylon cloves			Madagascar cloves		
Inoculation category	*Salmonella* Montevideo	*Salmonella* Typhimurium	*Salmonella* Weltevreden	*Salmonella* Montevideo	*Salmonella* Typhimurium	*Salmonella* Weltevreden
Low	−0.22	0.10	0.04	0.77	0.10	0.38
Medium	2.77	3.10	3.04	3.77	3.10	3.38
High	5.77	6.10	6.04	6.77	6.10	6.38

preenrichment methods. Five uninoculated negative controls were included in each trial.

Trypticase soy broth (TSB; Remel, Lenexa, KS) was used as the preenrichment medium. The 10- and 25-g cloves samples were mixed with 240 and 225 ml of TSB, respectively, in sterile Whirl-Pak filter bags. Three different preenrichment treatments were evaluated: (i) cloves were left in TSB and incubated at 35 ± 2°C for 24 ± 2 h during preenrichment (PreE1), (ii) the clove mixture was shaken vigorously for 30 s manually and the rinsate was then transferred to a fresh sterile Whirl-Pak bag and incubated at 35 ± 2°C for 24 ± 2 h (PreE2), and (iii) the clove mixture was prepared in the same way as for PreE2 except that it was shaken for 60 s (PreE3). After preenrichment, the BAM *Salmonella* culture method *(17)* was followed. The negative controls were uninoculated cloves of the same varieties as used for the inoculation experiments and were processed in the same manner as described above.

Validation of the newly designed detection and isolation method for *Salmonella* from cloves. To validate the effectiveness of this new method for isolation and detection of *Salmonella* from cloves, six trials were conducted to compare the new method with the current standard BAM method *(18)*. Each trial involved testing each of the two clove types against each of the three *Salmonella* serotypes, with 20 replicate test samples for the new method and another 20 test samples for the reference BAM method. Five negative controls, one environmental control, and two types of positive controls were included in each trial.

For the new method, 225 ml of TSB was added to 25 g of cloves in a sterile Whirl-Pak filter bag for each test sample and vigorously shaken manually for 60 s. The rinsate was then transferred to a fresh sterile Whirl-Pak bag and incubated at 35 ± 2°C for 24 ± 2 h. For the BAM method, 999 ml of TSB was added to 1 g of cloves in a sterile Whirl-Pak filter bag for each test sample, mixed well by swirling, and then incubated at 35 ± 2°C for 24 ± 2 h.

The positive broth control consisted of 250 ml of TSB with no cloves, and the positive sample (clove) control consisted of 225 ml of TSB with 25 g of cloves. These positive controls were spiked from one colony of the corresponding *Salmonella* serotype from a freshly streaked TSA plate and incubated at 35 ± 2°C for 24 ± 2 h in the same bag. The environmental control contained 250 ml of TSB in a sterile bag (no cloves and no *Salmonella*), which was incubated at 35 ± 2°C for 24 ± 2 h.

Microbiological assay. For cultural analysis in both evaluation and validation experiments, the preenrichment culture from all test samples was enriched in tetrathionate broth (BD) and Rappaport-Vassiliadis broth (prepared according to BAM formula) and incubated for 24 ± 2 h at 35 ± 2°C and 42 ± 0.2°C, respectively. Ten microliters of the selective enrichment cultures was then streaked onto xylose lysine deoxycholate (XLD; BD), bismuth sulfite (BS; BD), and Hektoen enteric (HE; BD) agar plates, incubated for 24 ± 2 h at 35 ± 2.0°C, and examined for typical colonies. When no colonies were observed on the BS plates, they were incubated for another 24 h. Isolated colonies were then confirmed according to the BAM *Salmonella* guidelines.

Pathatrix Auto System for immunomagnetic separation of *Salmonella* from cloves. Preenrichment test samples from the newly designed method were used to evaluate the effectiveness of the Pathatrix Auto System as an immunomagnetic separation method to isolate *Salmonella* from cloves. The Pathatrix *Salmonella* spp. Kit (APS50) was used, and all procedures followed the manufacturer's instructions. The captured Pathatrix bead suspensions were streaked onto selective agar plates (XLD, HE, and BS). These streaked plates were handled as described above.

Statistical analysis. A total of 432 test samples plus 30 control samples were created and analyzed in the method development part of the experiment. A total of 240 test samples plus 48 control samples were used in the validation part of the experiment. Data were grouped into categories and analyzed using SAS 9.4 (SAS Institute, Cary, NC). Fisher's exact test was used to determine significant differences between treatment groups at $\alpha = 0.05$.

RESULTS

The APCs for both Ceylon and Madagascar cloves were <100 CFU/25 g, thus these cloves had relatively low levels of bacterial contamination.

Development of a new detection and isolation method for *Salmonella* from cloves. Comparisons of *Salmonella* detection rates among the three preenrichment methods at three inoculation levels are shown in Table 2. All uninoculated negative controls for the six trials were negative for *Salmonella*. Treatments PreE2 and PreE3 produced significantly more *Salmonella*-positive results than did PreE1 ($P < 0.01$), especially at lower inoculation levels.

With PreE1, *Salmonella* was detected from only 1 of the 48 inoculated test samples at the low inoculation level (<1 log CFU/25 g), whereas treatments PreE2 and PreE3 resulted in 17 and 19 positive samples. At the medium inoculation level (~3 log CFU/25 g), PreE2 and PreE3 resulted in detection of *Salmonella* from 100% of the test samples, a rate four times higher than that for PreE1. At the high inoculation level (~6 log CFU/25 g), the *Salmonella* detection rate with PreE1 increased to 79.2% (38 of 48

TABLE 2. *Comparison of* Salmonella *detection rates from cloves for three preenrichment methods at three inoculation levels*[a]

Inoculation category (log CFU/25 g)[b]	Detection (no. of positive samples/no. tested)			Fisher's exact test[c]		
	PreE1	PreE2	PreE3	PreE1 vs PreE2	PreE1 vs PreE3	PreE2 vs PreE3
Low (<1)	1/48	17/48	19/48	<0.0001	<0.0001	0.8332
Medium (~3)	12/48	48/48	48/48	<0.0001	<0.0001	1
High (~6)	38/48	48/48	48/48	0.0006	0.0006	1
Total	51/144	113/144	115/144	<0.0001	<0.0001	0.8847

[a] PreE1, cloves were left in TSB and incubated at 35 ± 2°C for 24 ± 2 h during preenrichment; PreE2, clove-TSB mixture was manually shaken vigorously for 30 s, and rinsate was then transferred to a fresh sterile Whirl-Pak bag and incubated at 35 ± 2°C for 24 ± 2 h; PreE3, clove-TSB mixture was prepared the same as for PreE2 except that it was shaken for 60 s. All uninoculated controls were negative.
[b] See Table 1 for exact inoculation levels.
[c] Fisher's exact test was conducted using SAS 9.

samples), which is still significantly lower than the 100% rate with PreE2 and PreE3 ($P < 0.05$) based on Fisher's exact test. Treatment PreE1 produced only 51 *Salmonella*-positive results from the 144 tested samples, significantly fewer than the 113 of 144 detected with PreE2 and the 115 of 144 detected with PreE3 ($P < 0.05$, actually $P < 0.01$). In subsequent analysis, data from PreE1 were excluded, and we focused on analyzing results from treatments PreE2 and PreE3.

Results for comparing the effect of test sample size on the sensitivity of the *Salmonella* assay are presented in Table 3, including data from treatments PreE2 and PreE3. A sample size of 25 g resulted in relatively more positive results than did a sample size of 10 g at the low inoculation level, although the difference was not statistically significant. There was no significant difference at higher inoculation levels.

No significant differences in *Salmonella* detection rates were observed among the three serotypes tested (*Salmonella* Weltevreden, *Salmonella* Montevideo, and *Salmonella* Typhimurium) regardless of whether PreE2 and PreE3 data were considered separately or in combination (Table 4). No significant differences in *Salmonella* detection rates among the three serotypes were found between treatments PreE2 and PreE3 (Table 4), indicated that these two treatments were equally effective in detecting these *Salmonella* serotypes from cloves.

Between the two types of cloves studied, Madagascar and Ceylon, the detection rate of *Salmonella* was lower for the Ceylon (104 of 144 samples) than the Madagascar (124 of 144) cloves in the combined data from PreE2 and PreE3 (Table 5). Although the difference may be attributable to the slightly lower inoculation level in some of the Ceylon samples (Table 1), the fact that lower detection rate for the Ceylon cloves was more significant for the PreE2 treatment than for the PreE3 (Table 5) indicates that the PreE3 treatment was more effective when the microbial load was low.

TABLE 3. *Comparison of the effect of two sample sizes on* Salmonella *detection in cloves at three inoculation levels*

Inoculation category (log CFU/25 g)[a]	Detection (no. of positive samples/no. tested)		Fisher's exact test[b]
	10 g	25 g	
Low (<1)	16/48	20/48	0.5274
Medium (~3)	48/48	48/48	1.0000
High (~6)	48/48	48/48	1.0000
Total	112/144	116/144	0.6636

[a] See Table 1 for exact inoculation levels.
[b] Fisher's exact test was conducted using SAS 9.

Overall, the evaluation of the three treatments indicated that PreE3, in which cloves were removed from the preenrichment broth after 60 s of vigorous shaking, resulted in maximum detection rate of *Salmonella*. This treatment, with a sample size of 25 g, was designated as the new detection method and thus further validated and compared with the current BAM *Salmonella* detection method.

Validation of the newly designed detection and isolation method for *Salmonella* from cloves. The newly designed detection and isolation method, treatment PreE3 using 25 g of cloves, was compared with the FDA current standard BAM method in six trials, each with 120 test samples. The inoculation level for each trial was 2.40 to 4.31 log CFU/25 g, similar to the medium level in the method development experiments (Table 6). In all six trails, the new method produced more *Salmonella*-positive results than did the BAM method; all the differences were statistically significant. The newly designed method produced 16 to 20 positive results from 20 test samples in five of six trials (in one trial, 6 of 20 samples were positive), whereas the BAM method produced 0 to 6 positive results from 20 test samples in five of six trials (in one trial, 15 of 20 samples were positive) (Table 6).

Combining the data for all six validation trials for the two clove types and three *Salmonella* serotypes, the newly designed method detected *Salmonella* in 99 of 120 test samples, more than three times the rate with the reference BAM method (31 of 120 test samples). Therefore, our new method is significantly more sensitive ($P < 0.01$) than the reference BAM method for the detection and isolation of *Salmonella* from cloves (Table 6).

Efficiency of Pathatrix Auto System for detection of *Salmonella* from cloves. The Pathatrix Auto System was used for immunomagnetic separation and isolation of

TABLE 4. *Comparison of* Salmonella *detection rates from cloves for three* Salmonella *serotypes and two preenrichment treatments*

Preenrichment method[a]	Detection (no. of positive samples/no. tested)				Fisher's exact test[b]		
	Salmonella Montevideo	*Salmonella* Typhimurium	*Salmonella* Weltevreden	Total	SM vs ST	SM vs SW	ST vs SW
PreE2	38/48	35/48	40/48	113/144	0.6331	0.7944	0.3235
PreE3	38/48	36/48	41/48	115/144	0.6377	0.5939	0.3056
Total	76/96	71/96	81/96	228/288	0.4013	0.4550	0.1090
Fisher's exact test	1	0.8212	0.7876	0.8847			

[a] PreE2, clove-TSB mixture was shaken vigorously for 30 s manually, rinsate was then transferred to a fresh sterile Whirl-Pak bag and incubated at 35 ± 2°C for 24 ± 2 h; PreE3, clove-TSB mixture was prepared the same as for PreE2 except that it was shaken for 60 s.
[b] Fisher's exact test was conducted using SAS 9. SM, *Salmonella* Montevideo; ST, *Salmonella* Typhimurium; SW, *Salmonella* Weltevreden.

Salmonella. Selective agar test results from the captured Pathatrix beads are identical to results from our newly designed method (Table 6).

DISCUSSION

The current FDA BAM method for the detection and isolation of *Salmonella* from spices containing antimicrobial substances is designed to dilute these substances beyond their toxic concentrations. Cloves, for example, requires a sample/broth ratio of 1:1,000 *(18)* to dilute the antimicrobial agents. Thus, for a standard sample size of 25 g, 25 liters of preenrichment broth is needed per sample. To carry out this procedure in a laboratory is possible in some cases but not practical; in many laboratories it is impossible because of lack of incubator space. The alternative is to reduce the sample size to one or a few grams, which will result in a reduction of test sensitivity due to the substantial reduction of sample size and an inability to follow the FDA's sampling guidelines for this product *(18)*. Our newly designed method overcomes this problem by getting rid of inhibitory substances before preenrichment and reducing the regular sample/broth ratio to 1:9.

The results clearly indicated that removing cloves from the preenrichment broth before preenrichment can greatly improve the sensitivity of the detection method. Eugenol, a major component in the essential oil of cloves, has been bactericidal against *Salmonella* in many studies *(5, 6)*.

TABLE 5. *Comparison of* Salmonella *detection rates from two clove types with the PreE2 and PreE3 methods*

Preenrichment method[a]	Detection (no. of positive samples/no. tested)		Fisher's exact test[b]
	Ceylon cloves	Madagascar cloves	
PreE2	51/72	62/72	0.0415
PreE3	53/72	62/72	0.0954
Total	104/144	124/144	0.0055

[a] PreE2, clove-TSB mixture was manually shaken vigorously for 30 s, and rinsate was then transferred to a fresh sterile Whirl-Pak bag and incubated at 35 ± 2°C for 24 ± 2 h; PreE3, clove-TSB mixture was prepared the same as for PreE2 except that it was shaken for 60 s.
[b] Fisher's exact test was conducted using SAS 9.

Incubation of cloves in the preenrichment broth at around 35°C facilitates the release of eugenol into the medium and can inhibit the growth of bacteria. This release was observed in the PreE1 treatment, in which the cloves were left with the preenrichment incubation for 24 h. The isolation rate of *Salmonella* from the PreE1 treatment was close to 0% at an inoculation level below 1 log CFU/25 g and was only 25% at an inoculation level around 3 log CFU/25 g. In contrast, the new method PreE3 allows an interaction time of only 60 s between the cloves and the preenrichment medium. Despite the transient, yet vigorous, mixing of sample and broth, this method allowed isolation of *Salmonella* from the spice sample and detection of *Salmonella* from 40% of the test samples at the low inoculation level of <1 log CFU/25 g and from 100% of the test samples at the medium inoculation level of 3 log CFU/25 g (Table 2). These significant differences indicate that removing the cloves from the preenrichment broth reduced or eliminated the antimicrobial substances in cloves and facilitated the growth and detection of *Salmonella*.

In addition to the standard sample size of 25 g, we also evaluated a sample size of 10 g to determine whether a smaller sample size would further reduce the presence of antimicrobial substances in the growth medium and hence increase the sensitivity of *Salmonella* detection. However, our results have shown that a smaller amount of spices in the broth did not improve the test results but rather reduced the isolation of *Salmonella* at low inoculation levels (Table 3). Likewise, a shorter spice-broth mixing time of 30 s did not improve the test results compared with mixing for 60 s (Tables 5). Based on these findings, we designed the alternative detection and isolation method, including these key procedures: sample size of 25 g, mixing time of 60 s, and sample/broth ratio of 1:9. With this method, even larger sample sizes, i.e., more than 25 g, could be used just like most available protocols for vegetables, fruits, and other foods.

The validation of our newly designed method was conducted by parallel comparison with the BAM method (Table 6). Each of the six validation trials indicated that the detection rate of *Salmonella* using the BAM preenrichment method was significantly lower than that using the newly designed method. Based on the data of all six validation trails with both clove types and the three *Salmonella*

TABLE 6. *Validation of the newly designed method for the detection of* Salmonella *from cloves compared with the reference FDA BAM methods*[a]

Clove variety	*Salmonella* serotype	Inoculation level (log CFU/25 g)	No. of positive samples/no. tested			Fisher's exact test[b]
			New method	Pathatrix	Reference method (BAM)	
Ceylon	Montevideo	3.30	20/20	20/20	6/20	<0.0001
	Typhimurium	3.60	16/20	16/20	5/20	0.0012
	Weltevreden	4.31	20/20	20/20	15/20	0.0471
	Subtotal		56/60	56/60	26/60	<0.0001
Madagascar	Montevideo	2.68	6/20	6/20	0/20	0.0202
	Typhimurium	3.09	19/20	19/20	3/20	<0.0001
	Weltevreden	2.40	18/20	18/20	2/20	<0.0001
	Subtotal		43/60	43/60	5/60	<0.0001
	Total		99/120	99/120	31/120	<0.0001

[a] All uninoculated controls were negative.
[b] Fisher's exact test was conducted using SAS 9.

serotypes, the alternative method detected *Salmonella* from 99 of 120 inoculated test samples, whereas the reference BAM method detected only 31 of 120. With the big increase in sensitivity, the newly designed method is also much more similar to commonly used methods, and it saves laboratory space and resources compared with the reference BAM protocol.

Salmonella detection results from the Pathatrix Auto System followed by selective agar culture completely matched the culture results from our newly designed detection method (Table 6), which indicated that the Pathatrix Auto System could be used to effectively detect *Salmonella* directly from preenrichment samples prepared by our new method, saving the time and labor of selective broth enrichment. The Pathatrix Auto System has been previously evaluated as a rapid method for isolation of *Salmonella* from foods, mostly linked to downstream detection methods such as selective agar culture or PCR *(11, 23–25)*. In two studies, the Pathatrix assay with selective agar was less effective than the traditional culture method *(11, 24)*. In one study, the Pathatrix assay with XLD agar was more sensitive than the BAM method for tomato but not for ground beef. Nevertheless, use of the Pathatrix method reduced overall assay time compared with the traditional BAM method *(25)*.

In conclusion, our newly designed detection and isolation method, in which cloves (25 g) are removed from preenrichment broth after 60 s of vigorous shaking, improved the sensitivity for detecting *Salmonella* from cloves from 3 to <1 log CFU/25 g in comparison with the method of leaving the cloves in the broth. This alternative method was significantly superior to the current FDA BAM method in all six validation trials and could replace the current BAM method. Further validation studies of this method are underway for other spices with antimicrobial substances.

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