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# Direct Quantitation and Detection of Salmonellae in Biological Samples without Enrichment, Using Two-Step Filtration and Real-Time PCR

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**A new two-step filtration protocol followed by a real-time PCR assay based on SYBR green I detection was developed to directly quantitate salmonellae in two types of biological samples: i.e., chicken rinse and spent irrigation water. Four prefiltration filters, one type of final filter, and six protocols for recovery of salmonellae from the final filter were evaluated to identify an effective filtration protocol. This method was then combined with a real-time PCR assay based on detection of the *invA* gene. The best results were obtained by subsequent filtration of 100 ml of chicken rinse or 100 ml of spent irrigation water through filters with pore diameters of >40 μm to remove large particles and of 0.22 μm to recover the *Salmonella* cells. After this, the *Salmonella* cells were removed from the filter by vortexing in 1 ml of physiological saline, and this sample was then subjected to real-time quantitative PCR. The whole procedure could be completed within 3 h from sampling to quantitation, and cell numbers as low as $7.5 \times 10^2$ CFU per 100-ml sample could be quantified. Below this limit, qualitative detection of concentrations as low as 2.2 CFU/100 ml sample was possible on occasion. This study has contributed to the development of a simple, rapid, and reliable method for quantitation of salmonellae in food without the need for sample enrichment or DNA extraction.**

*Salmonella* is one of the most common causes of food-borne disease (27), with 40,000 reported annual cases of salmonellosis and an even higher number of estimated cases in the United States (data available at www.cdc.gov). In order to minimize risks for the consumer, microbial auditing of food is increasingly being applied. For this reason, the number of rapid test methods for *Salmonella* has grown rapidly in the last decade. PCR and real-time PCR have become powerful tools for the detection of pathogens in food. Many different PCR assays have been developed for *Salmonella*, all with different specificities, accuracies, and detection limits (13, 15, 35). The most recent assays, with detection in 12 to 20 h, have drastically improved the speed of the detection process compared to that of culture-based methods (3, 10). However, due to the potential for very low levels of salmonellae in foods and standards requiring detection of 1 CFU of salmonellae in food, all these methods include a significant enrichment time that limits the ability for same-day analysis. Also, even though real-time PCR allows for quantitation of targets, after enrichment the number of cells present has generally been changed in an unpredictable manner, making quantitation of the initial amount of target difficult. For these reasons, it would be considered an improvement to be able to detect and, if possible, quantitate (low) levels of salmonellae in foods without the need for enrichment.

Aside from concentrating the target, sample treatments are performed prior to PCR in order to remove PCR inhibitors or to improve the homogeneity of samples (19). There are currently several methods for detection of bacteria in biological samples without enrichment. Methods for direct detection of, for example, *Salmonella* or *Campylobacter* in fecal or cecal samples have recently been published (1, 6, 21), and another study described direct quantitation of *Oenococcus oeni* in wine (18). The common aspect between those studies was that the bacterial concentrations in the samples were high, and enrichment was therefore unnecessary. A recent study used a new sample treatment called floatation prior to real-time PCR, allowing direct quantitation of *Yersinia enterocolitica* in meat samples (33). This method allowed detection of $10^2$ CFU/ml in pork juice samples. Nonetheless, the main limitation of this method, currently, is the small sample volume (1 to 2 ml) that is used for analysis. For food sampling, often samples as large as 10 g or 25 g in 100 or 250 ml of solution are used. As the ideal goal is to detect one cell in such a sample (24), it is statistically much easier to detect that single cell when the whole sample volume can be used for analysis. To date, only a very small number of studies have successfully used larger samples for direct detection of very low concentrations of pathogens (between $10^1$ and $10^2$ CFU per gram of sample) in food. One study used centrifugation, DNA extraction, PCR amplification, and amplicon hybridization for the detection of *Salmonella* and *Listeria* in yogurt and cheese (26), whereas another used centrifugation, filtration, and enzymatic digestion followed by PCR for the detection of *Listeria monocytogenes* in cheese homogenates (28).

It has been suggested that if bacteria could be easily separated, purified, and concentrated from a biological sample, the application of rapid detection technologies such as PCR and real-time PCR could be expanded (2, 25). Recent studies showed that in the case of mildly PCR-inhibitory samples such as, for example, chicken rinse and (irrigation) water, the use of alternative DNA polymerases could greatly reduce the negative effects of sample components and background flora (12). These results suggest that when working with those samples,

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nonspecific concentration of the sample can be combined with real-time PCR. One such method that has been applied to concentrate or separate pathogens from food is filtration (26). Several studies have described the use of a crude filtration step prior to additional treatments such as enzymatic treatment and centrifugation (9, 28, 31). The goal of this study was to develop and evaluate a two-step filtration procedure followed by quantitative real-time PCR for the detection of *Salmonella* in different biological samples containing low numbers of the pathogen. Chicken skin rinses and spent irrigation water were used as the model systems.

## MATERIALS AND METHODS

**Bacteria and culture methods.** *Salmonella enterica* serotype Typhimurium C1058 and DT108, *Salmonella enterica* serotype Enteritidis C1016, and *Salmonella enterica* serotype Hadar SHA were obtained from the Canadian Research Institute for Food Safety culture collection. Strain C1058 was used as a model strain in all experiments, but the final protocol was confirmed with the other strains. Strains were grown overnight in buffered peptone water (Oxoid, Basingstoke, Hampshire, United Kingdom) at 37°C with shaking at 200 rpm. Cell counts were conducted on LB agar (Becton, Dickinson and Company, Sparks, MD) or, for specific growth, on brilliant green agar (modified) (Oxoid) and/or bismuth sulfite agar (Oxoid) with incubation at 37°C for 24 and 48 h. DNA from strain C1058 for amplification efficiency testing of the prefiltrates was purified from liquid cultures by using a MagnaPure automated DNA purification system (Roche Diagnostics, Mannheim, Germany). DNA concentrations were determined spectrophotometrically.

**Biological samples.** Chicken was bought at a local supermarket, and chicken skin rinse samples were made by adding 90 ml of physiological saline to 10 g of chicken skin, followed by homogenization in a stomacher for 30 seconds. Spent irrigation water was produced by sprouting 50 g of mung beans for 24 h (procedure adapted from that in reference 5). The beans were obtained from local supermarkets. The beans were soaked for 3 h in sterile, deionized water. After this, the beans and sprouts were rinsed three times over the next 21 h with water volumes equal to the weight of the bean sprouts. At 24 h, the bean sprouts were rinsed a final time, and the water was collected and used as spent irrigation water samples.

**Real-time PCR conditions.** A real-time PCR assay was developed based on an existing primer set coding for a 284-bp region of the *invA* gene (20). The PCR mixture consisted of 0.75 U *Tth* DNA polymerase (Roche Diagnostics); 1× *Tth* DNA polymerase buffer (Roche Diagnostics); 0.4 μM of each primer; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 4 mM total $MgCl_2$ concentration; 1 μl of 10,000-times-diluted SYBR green I (Roche Diagnostics); and 4 μl of sample in a total volume of 20 μl. *Tth* DNA polymerase was chosen due to its greater resistance to PCR inhibitors (12). Therefore, *Tth* DNA polymerase and its buffer were used for all experiments during this study. Each amplification cycle started with a denaturation step of 1 min at 95°C; followed by 40 cycles of 0.1 s of denaturation at 95°C, 5 s of annealing at 60°C, and 25 s of elongation at 72°C; followed by a single fluorescent measurement and 25 s of final elongation. Amplification was followed by a melting curve analysis between +65°C and +95°C and, finally, a cooling step for 1 min at +40°C. Positive product peaks appeared between 87 and 89°C. During amplification, the fluorescence was measured in channel F1. The quantitation data, in terms of crossing point (Cp) values (Cp is expressed as a fractional cycle number and is the intersection of the log-linear fluorescence curve with a threshold crossing line), were determined using the second derivative method of the LightCycler software, version 5.3 (Roche Diagnostics). After amplification, the Cp values of the samples were plotted against the log of the initial DNA concentration. After this, linear regression was performed to calculate the slope of the plot of Cp versus log initial DNA concentration, using the Cp values in the linear range. From this slope, the amplification efficiency (AE) was calculated using the equation $(10^{-1/\text{slope}}) - 1$ (8). The PCR efficiency was used as a reference to determine the level of PCR inhibition present in the sample.

**Prefiltration.** Filtration was performed in two steps: a crude prefiltration step to remove larger sample particles and a second filtration to recover the target bacteria. Four different filters were tested for the prefiltration step: Whatman no. 2 (>8 μm) and no. 4 (20 to 25 μm) filter papers (Whatman International Ltd., Maidstone, United Kingdom), four layers of cheesecloth (American Fiber and Finishing Inc, Albemarie, NC), and VWR qualitative filter paper grade 417 (>40 μm) (VWR Scientific Products, West Chester, PA). The filters were folded and placed in a funnel holder, and filtrate was collected for bacterial cell counts and PCR inhibition evaluations. To evaluate the performances of different prefilters, the time to filter 100 ml chicken rinse, the amplification efficiency in the sample, and the recovery of the cells in the filtrate were measured. The amplification efficiency in the sample was used as a tool in order to check the PCR inhibition in the samples after crude filtration. Tenfold dilutions of *Salmonella* DNA between 1 mg/ml and 1 fg/ml (or $2 \times 10^{11}$ to 0.2 genomic *Salmonella* DNA copies per ml) (14) were spiked into the different filtrates and were used to obtain standard curves. The amplification efficiency was calculated from these standard curves. All measurements were carried out as independent duplicate experiments. The recovery of the cells in the filtrate was tested by plating a dilution series of prefiltered samples that had been initially spiked with $3.70 \times 10^7 \pm 0.02 \times 10^7$ CFU/100 ml of *Salmonella*.

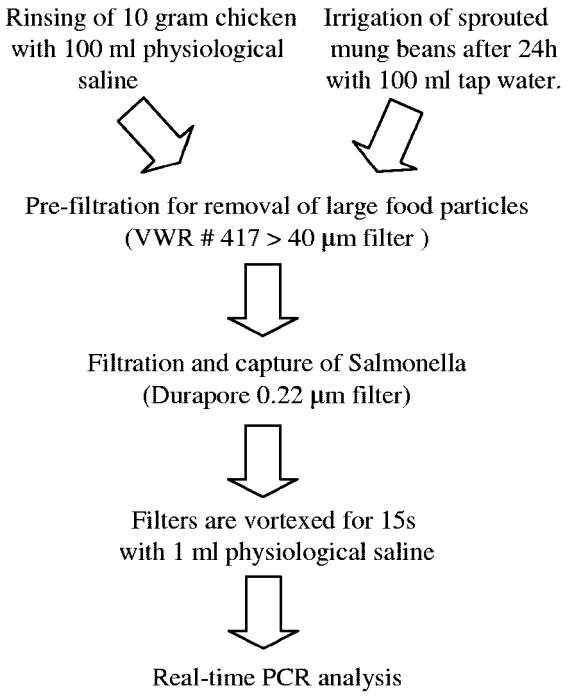


FIG. 1. Schematic overview of the developed two-step filtration protocol followed by real-time PCR analysis.

**Target recovery from filters.** For vacuum filtration in the second step, Durapore 0.22-μm membrane filters (Millipore Corporation, Bedford, MA) were chosen. After retention of the target cells on the Durapore filters, seven tests within four different strategies were chosen to remove the target cells or their DNA from the filters. The first strategy included placement of the filter in a 15-ml centrifuge tube and addition of 1 ml physiological saline, followed by vortexing for either 15, 30, or 60 s. The second strategy, loosely based on a study by Kirk and Rowe (7), included placement of the filter in a 15-ml tube, addition of 1 ml of physiological saline, and sonication in a sonic cleaner for 1 min. The third treatment strategy was based on cell lysis directly on the filter, as described by Wu and Kado (34). This treatment included placement of the filter in a 15-ml tube and addition of 1 ml of lysis buffer (0.25% Triton X-100, 10 mM Tris [pH 8.0], and 1 mM EDTA). The final strategy included application of traditional chloroform extraction and ethanol precipitation directly from the filter. Analysis of the recovery was performed using quantitation by real-time PCR of the cell numbers in the sample prior to filtration (without prefiltration) and after filtration and the selected recovery treatment (22).

**Final filtration experiments.** The final protocol used for analysis of spiked and naturally contaminated samples after optimization is described in Fig. 1. In some cases, the final filter was clogged by the sample, slowing down the procedure. It was possible to speed up the process by removing the first filter, changing to a fresh filter, and vortexing all filters in the same final 1 ml without interfering with the results (up to three filters were used and processed together). Spiking concentrations were confirmed with plate counts, and plate counts also were performed on the unspiked samples. After filtration, *Salmonella* concentrations were quantified using real-time PCR. Due to possible remaining PCR inhibition

TABLE 1. Comparison of four crude filtration methods

| Filter type | Recovery of cells in filtrate (%)[a] | Amplification efficiency in filtrate samples[b] | Filtration time[c] |
|---|---|---|---|
| Cheesecloth | 97.1 ± 1.9 | 1.15 | <1 min |
| VWR no. 417 | 79.1 ± 6.0 | 0.93 | 29 min |
| Whatman no. 2 | 54.3 ± 21.0 | 1.07 | 5 h 34 min |
| Whatman no. 4 | 58.5 ± 0.8 | 0.94 | 3 h 1 min |

[a] As determined by plate counts of independent duplicate samples. Results are means and standard deviations.
[b] Two independent experiments using purified DNA were run, and the duplicate data were used to create one standard curve. This standard curve was used to calculate the amplification efficiency in the samples.
[c] Time for filtration of 100 ml of chicken rinse sample through one filter. Results are averages of at least duplicate data.

TABLE 2. Comparison of methods to recover cells or DNA from filters

| Recovery method | Recovery (%)[a] |
|---|---|
| Vortexing for 15 s | 103 ± 7 |
| Vortexing for 30 s | 80 ± 15 |
| Vortexing for 1 min | 100 ± 30 |
| Sonication | 94 ± 26 |
| Sonication in lysis buffer | 62 ± 30 |
| Direct lysis from filter | 93 ± 36 |
| DNA extraction from filter | 29 ± 13 |

[a] Results are averages and standard deviations of quadruplicate independent measurements obtained by real-time PCR (standard curve for *Salmonella* cells: $y = -3.8648x + 44.254$; $r^2 = 0.9628$).

in some spent irrigation water samples, all samples were also diluted 10-fold and analyzed to confirm the data.

**Statistical analysis.** One-way analysis of variance, using MS Excel (Microsoft Corporation, Seattle, WA), was performed to find significant differences between treatments. A $P$ value of below 0.05 indicated a significant difference.

## RESULTS AND DISCUSSION

**Real-time PCR assay.** The real-time PCR assay used in this study was optimized and had amplification efficiencies of 1.00 ($r^2 = 0.998$) when purified *Salmonella* DNA was used as a target and 0.82 ($r^2 = 0.989$) when whole *Salmonella* cells were used (data not shown). The quantitation limit was determined through testing 10-fold dilutions of *Salmonella* cells, and quantitation of levels as low as 2 *Salmonella* CFU per PCR was established (data not shown). These results were similar to those of other published real-time PCR assays using the same primers, such as a molecular beacon assay detecting 1 cell per assay (10) or 10 cells per assay using hybridization probes (17). Using whole cells, a linear range of amplification (or quantifiable range) was established from $5 \times 10^2$ CFU/ml to $5 \times 10^8$ CFU/ml. Levels below this range were detected on occasion, but standard deviations were too great to allow accurate quantitation. The specificity of the *invA* primers was not tested, as several studies have evaluated their suitability for specific detection of salmonellae (20, 35). In order to formulate the PCR assay to reduce possible inhibition and to improve quantitation, the alternative polymerase *Tth* was used, as previous studies have indicated that this enzyme is less sensitive to inhibitors present in chicken rinse (12) and is well suited for quantitation experiments (32). To prepare the PCR assay for use in routine diagnostics, an internal amplification control will need to be incorporated into the assay, and the use of sequence-specific probes also should be considered (5).

**Filtration.** In the two-step filtration setup, the first step is intended to remove crude food particles from the sample and thus limit PCR inhibition and clogging of the next filter while still allowing bacterial targets to pass through the filter. Samples processed through three different filter papers with different pore sizes and one cheesecloth were analyzed for PCR inhibition after filtration, the percentage of target cells in the filtrate, and the time of filtration. The results showed that the cheesecloth retained significantly fewer cells than the VWR and the Whatman no. 4 filter ($P = 0.025$ and $P = 0.001$, respectively), whereas the other filters retained up to 46% of the cells (Table 1). Filtration through the cheesecloth also was more rapid ($P < 0.001$) than the other methods, with a filtration time of less than 1 minute, compared to up to several hours for other filters. Several other studies have applied large-pore filters, similar to cheesecloth, to remove large particles before further treatment (28, 30). To evaluate the PCR inhibition in the filtrate after this first filtration, the amplification efficiency for DNA in the sample was determined and compared with those in water (AE = 1.00) and in unfiltered chicken rinse samples (AE = 1.33). When a PCR performs optimally and amplification is exponential, the AE is 1.00. When the efficiency of the reaction goes down, this signifies that the amplification is inhibited. Theoretically, values higher than 1 are not possible, but due to the standard deviation resulting from inhibition and/or a limited number of data points, values above 1 are found as well. The results showed that the cheesecloth did reduce the PCR inhibition compared to chicken rinse but clearly less than the other filters (Table 1). Furthermore, because of the lower retention by the cheesecloth, the resulting filtrate clogged the filter in the second step rapidly and prevented concentration of the bacteria (data not shown). Of the three remaining filters, the VWR filter performed the best and was chosen for prefiltration, due to its higher speed and lower retention compared to the two Whatman filters.

The second step included capture of the target *Salmonella* on a filter and recovery of the cells or the DNA from the filter. Oyofo and Rollins (16) investigated the use of different filters in combination with PCR. They concluded that five of nine studied filters completely inhibited PCR, while the four others allowed amplification at different levels. It was suggested that this inhibition was due to the binding of DNA to the filter membranes. Based on these data, 0.22-μm Durapore filters were chosen for this study. After filtration through these filters, fewer than 0.3% of the cells were found in the filtrate. To optimize the recovery of the cells or their DNA from the filters, seven different recovery methods were compared (Table 2). The highest recovery rate (combined with lowest standard deviation) was found after vortexing for 15 seconds. Analysis of variance showed that the recovery of 103% ± 7% was significantly higher than that with filters vortexed for 30 seconds or after using either sonication in lysis buffer or DNA extraction directly from the filters ($P > 0.05$). High recovery rates were also found after vortexing for 1 min, sonicating, or performing direct lysis from the filter ($P = 0.83$, $P = 0.51$, and $P = 0.84$, respectively, compared to vortexing for 15 seconds). The results obtained following sonication were similar to those of previous studies on *Campylobacter* (7). Nonetheless, due to the

TABLE 3. Quantitation of *Salmonella* in artificially contaminated chicken rinse and irrigation water samples by filtration and real-time PCR

| Sample type | *Salmonella* concn[a] | |
|---|---|---|
| | Before filtration as determined by plating (CFU/100 ml of sample) | After filtration as determined by real-time PCR (CFU/ml) |
| Chicken rinse | $3.4 \times 10^5 \pm 0.8 \times 10^5$ | $6.9 \times 10^5 \pm 1.1 \times 10^5$ |
| | $3.4 \times 10^4 \pm 0.8 \times 10^4$ | $4.6 \times 10^4 \pm 2.9 \times 10^4$ |
| | $2.2 \times 10^3 \pm 0.1 \times 10^3$ | $7.3 \times 10^3 \pm 2.5 \times 10^3$ |
| | $2.2 \times 10^2 \pm 0.1 \times 10^2$ | 3/3[b] |
| | $2.2 \times 10^1 \pm 0.1 \times 10^1$ | 2/3[b] |
| | $2.2 \pm 0.1$ | 1/3[b] |
| | 0 | 0 |
| Spent irrigation water | $7.5 \times 10^4 \pm 3.0 \times 10^4$ | $3.8 \times 10^4 \pm 3.9 \times 10^4$ |
| | $7.5 \times 10^3 \pm 3.0 \times 10^3$ | $8.0 \times 10^3 \pm 2.8 \times 10^3$ |
| | $7.5 \times 10^2 \pm 3.0 \times 10^2$ | $6.8 \times 10^2 \pm 2.8 \times 10^2$ |
| | $7.5 \times 10^1 \pm 3.0 \times 10^1$ | 1/3[b] |
| | $7.5 \pm 3.0$ | 0/3[b] |
| | 0 | 0 |

[a] Except as noted, all data are means and standard deviations from triplicate analysis.

[b] Real-time PCR showed quantifiable data within the linear range of amplification of $5 \times 10^2$ CFU/ml to $5 \times 10^8$ CFU/ml. Below this range positive results were observed by melting curve analysis, but they are of a qualitative nature and are expressed as number positive/number of samples tested.

TABLE 4. Detection of *Salmonella* in naturally contaminated chicken rinse and irrigation water samples by filtration and real-time PCR

| Sample type | No. of samples | *Salmonella* concn | |
|---|---|---|---|
| | | Before filtration as determined by plating (CFU/100 ml of sample) | After filtration as determined by real-time PCR (CFU/ml) |
| Chicken rinse | 18 | 0 | 6/18[a] |
| | 1 | 3,500 | 1,134 |
| Irrigation water | 20 | 0[b] | 5/20[a] |

[a] Real-time PCR showed quantifiable data within the linear range of amplification of $5 \times 10^2$ CFU/ml to $5 \times 10^8$ CFU/ml. Below this range positive results were observed by melting curve analysis, but they are of a qualitative nature and are expressed as number positive/number of samples tested.

[b] No positive *Salmonella* colonies could be identified on brilliant green agar and bismuth sulfite agar, possibly due to a large amount of background flora present in the irrigation water samples.

observed high standard deviations (from 26% to 36%), these methods were not chosen for the final protocol. Finally, an additional reason to select the vortexing method was that it offered the possibility of performing plate counts and classical analysis of the target should this be desired. In total, on average the whole two-step filtration procedure could be performed within 75 min.

**Quantitation of salmonellae in biological samples.** In the final part of this study, the developed two-step filtration system was combined with a real-time quantitative PCR assay (Fig. 1). Although the method was in principle designed for concentration of salmonellae from chicken skin rinses, the final protocol was also tested on another dilute sample: spent irrigation water from bean sprouts. Bean or seed sprouts have been frequently implicated in outbreaks involving *Salmonella* (11, 29). The first experiment using the combined methods aimed to quantitate different concentrations of *Salmonella* in artificially contaminated *Salmonella*-free chicken rinse and irrigation water samples (Table 3). Results showed that concentrations as low as $2.2 \times 10^2 \pm 0.1 \times 10^2$ CFU per 100-ml sample could be positively identified in all cases, and numbers equal to and higher than $7.5 \times 10^2 \pm 3.0 \times 10^2$ could be quantified using this protocol. Low levels (below 250 CFU/sample) were detected occasionally, which can be expected since levels below 250 CFU/ml in the final sample after filtration (which means 1 CFU/PCR sample) have a detection probability of below 1. Although all numbers quantified with real-time PCR were of the same order of magnitude as the numbers added to the original sample, quantified numbers varied from the numbers calculated with plate counts (Table 3). Deviations might be explained by variations in recovery in the two filtration steps or detection of DNA from injured or dead cells.

The second experiment detected *Salmonella* in naturally contaminated samples (Table 4). The results showed that of the 19 tested chicken rinse samples, 12 were negative by both methods; 6 samples had amounts undetectable by plate counts prior to filtration and were positive by real-time PCR, showing Cp values below the quantifiable range. A final sample showed *Salmonella* concentrations within the quantifiable range. This sample was positively identified and quantified. No spent irrigation water samples had numbers high enough to be quantified directly in the sample by plate counts. Still, of the 20 samples, 5 showed low positive results with the filtration and real-time PCR protocol but were negative in plate counts. This may be explained by the concentration of salmonellae during the filtration procedure but could also be due to nonoptimal growth of the *Salmonella* on the solid growth medium, interference from background flora on the solid growth medium, or detection of DNA originating from dead cells (23).

In summary, a new filtration technique has been developed, which, when combined with real-time PCR, can detect levels as low as 220 CFU of *Salmonella* in 100-ml chicken rinse samples. All samples with concentrations higher than 750 CFU/100 ml sample were positively quantified, and samples with concentrations as low as 2.2 CFU/100 ml were qualitatively detected on occasion. Future research should focus on further concentrating the sample. This can be done by further reducing the resuspension volume after filtration and/or by increasing the sample volume applied to filtration. As the current method can be performed within 3 h from sampling to final results, is cost-effective, and is able to detect as little as 220 CFU of *Salmonella* cells within a 100-ml sample, it offers a sensitive, rapid, simple quantitative alternative to existing direct detection methods (26, 28). Although the current detection levels might not be as low as the detection levels of 5 cells per 25 g (17) to 3 CFU/ml *Salmonella* in poultry samples (4) which are recorded when using PCR combined with enrichment, this study has shown that detection without culture enrichment or DNA extraction is approaching the same levels of sensitivity and reproducibility as culture-based methods.

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