APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2009, p. 5321–5327 Vol. 75, No. 16
0099-2240/09/$08.00+0 doi:10.1128/AEM.02422-08

Development of a Cell Culture Method To Isolate and Enrich *Salmonella enterica* Serotype Enteritidis from Shell Eggs for Subsequent Detection by Real-Time PCR[▽]

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Received 22 October 2008/Accepted 24 May 2009

Salmonella enterica **serotype Enteritidis is a major cause of nontyphoidal salmonellosis from ingestion of contaminated raw or undercooked shell eggs. Current techniques used to identify *Salmonella* serotype Enteritidis in eggs are extremely laborious and time-consuming. In this study, a novel eukaryotic cell culture system was combined with real-time PCR analysis to rapidly identify *Salmonella* serotype Enteritidis in raw shell eggs. The system was compared to the standard microbiological method of the International Organization for Standardization (Anonymous, Microbiology of food and animal feeding stuffs—horizontal method for the detection of *Salmonella*, 2002). The novel technique utilizes a mouse macrophage cell line (RAW 264.7) as the host for the isolation and intracellular replication of *Salmonella* serotype Enteritidis. Exposure of macrophages to *Salmonella* serotype Enteritidis-contaminated eggs results in uptake and intracellular replication of the bacterium, which can subsequently be detected by real-time PCR analysis of the DNA released after disruption of infected macrophages. Macrophage monolayers were exposed to eggs contaminated with various quantities of *Salmonella* serotype Enteritidis. As few as 10 CFU/ml was detected in cell lysates from infected macrophages after 10 h by real-time PCR using primer and probe sets specific for DNA segments located on the *Salmonella* serotype Enteritidis genes *sefA* and *orgC*. *Salmonella* serotype Enteritidis could also be distinguished from other non-serogroup D *Salmonella* serotypes by using the *sefA*- and *orgC*-specific primer and probe sets. Confirmatory identification of *Salmonella* serotype Enteritidis in eggs was also achieved by isolation of intracellular bacteria from lysates of infected macrophages on xylose lysine deoxycholate medium. This method identifies *Salmonella* serotype Enteritidis from eggs in less than 10 h compared to the more than 5 days required for the standard reference microbiological method of the International Organization for Standardization (Microbiology of food and animal feeding stuffs—horizontal method for the detection of *Salmonella*, 2002).**

Nontyphoidal salmonellosis is an invasive intestinal disease contracted predominately by ingestion of food contaminated with serotypes of the gram-negative bacterial species *Salmonella enterica*. Gastroenteritis caused by *Salmonella* spp. represents a large portion of the natural food-borne illnesses that occur worldwide each year. Bacterial virulence is established in part by the bacterium's ability to invade and survive within host cells (20). *S. enterica* is capable of survival within a wide array of host cells, including epithelial cells, dendritic cells, and macrophages in both animal and cell culture models (16, 17, 18, 19). However, survival in macrophages is required for initiation of systemic infection (24). Two chromosomal pathogenicity islands, SPI-1 and SPI-2, which are present in all *Salmonella enterica* serotypes, are essential for the invasion of epithelial cells and intracellular replication in macrophages, respectively (13, 14).

There are currently over 2,500 distinct serotypes of *S. enterica* (http://www.pasteur.fr/sante/clre/cadrecnr/salmoms/WKLM_2007.pdf). Of these, *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium are most commonly associated with food-borne illness in humans (4). Raw and undercooked shell eggs have been implicated as vehicles for the transmission of both of these serotypes of *Salmonella enterica* (9, 38). However, *Salmonella* serotype Enteritidis infection has been more frequently linked to shell egg consumption, whereas *Salmonella* serotype Typhimurium infection is more often associated with the consumption of contaminated chicken meat (8). Of the 309 documented outbreaks of *Salmonella* serotype Enteritidis in the United States from 1990 to 2001, 241 were attributed to the consumption of raw or undercooked eggs (6). *Salmonella* serotype Enteritidis phage types 4, 8, and 13 have been implicated in the majority of salmonellosis cases from the consumption of egg products (5). In addition, *Salmonella* serotype Enteritidis is able to colonize laying hen reproductive organs and developing eggs and has been shown to persist in eggs after they have been laid (23).

A variety of methods have been developed in order to expedite the detection of salmonellae in eggs, including GeneQuence DNA hybridization, PCR analysis, and enzyme-linked immunosorbent assay (3, 27, 37). However, these methods require lengthy enrichment steps prior to the application of the respective methods. Real-time PCR (RT-PCR) is a promising new method currently used for detection of a wide variety of bacterial pathogens in food matrices (12, 15, 22, 34, 40). However, this technique can be ineffective for the detection of *Salmonella* serotype Enteritidis in foods such as eggs due to the presence of PCR-inhibitory components (41).

In this study, we developed a novel detection system to allow

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▽ Published ahead of print on 26 June 2009.

TABLE 1. Primers and probes used in this study

Primer or probe name	Sequence (5′ to 3′)	Final reaction mixture concn (nM)	Source or reference
SEFA-F (forward)	GGCTTCGGTATCTGGTGGTGTG	50	37
SEFA-R (reverse)	GTCATTAATATTGGCTCCCTGAATA	900	37
SEFA-P (probe)	CCACTGTCCCGTTCGTTGATGGACA	250	37
ORGC-F (forward)	CTTTATGATGCATTCTACCAACGACTG	100	This work
ORGC-R (reverse)	CCGAATCACCACTGTTAGGA	100	This work
ORGC-P (probe)	CGCTTCCTGAGTCAGCCTCTTCTGAAACG	100	This work

for the specific identification of viable *Salmonella* serotype Enteritidis in raw shell eggs. The method developed is based on the ability of *Salmonella* to invade and replicate within macrophages as part of its life cycle within a host. In theory, cultured eukaryotic cell lines exposed to *Salmonella*-contaminated foods will allow the penetration and replication of *Salmonella* while confining food particles and noninvasive bacteria to the extracellular environment, allowing the isolation and enrichment of intracellular *Salmonella* for subsequent detection by commercially available techniques, such as RT-PCR. In practice, a suitable mammalian cell monolayer is exposed to a particular food matrix suspected of harboring salmonellae. The exposure is promoted for sufficient time to allow cell contact and engulfment of salmonellae. The mammalian cell monolayer is then washed sufficiently to remove the food matrix and extracellular microorganisms. The infected cell monolayer is reconstituted with fresh medium and further incubated to allow for intracellular multiplication of *Salmonella* (postinfection). After the infection is terminated, the culture medium is discarded, the infected cells are disrupted, and the DNA present in the resultant lysates is analyzed by RT-PCR using primers and probes specific for unique *Salmonella* DNA sequences. We utilized this method for the presumptive and confirmatory identification of *Salmonella* serotype Enteritidis in raw shell eggs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella* serotype Typhimurium (phage type DT104) and *Salmonella* serotype Enteritidis (phage types 4, 8, and 13) were used in cell culture infection experiments and routinely grown on modified Luria-Bertani (LB) broth or agar and xylose lysine deoxycholate (XLD) agar at 37°C. All *Salmonella* strains used (see Table 2) were obtained from the FDA facility (College Park, MD) and grown on modified LB agar at 37°C and used for primer and probe specificity testing. *Yersinia*, *Listeria*, *Vibrio*, *Enterobacter*, and *Escherichia* strains (see Table 3) were grown in heart infusion broth at 37°C and used for primer and probe specificity testing. *Clostridium botulinum*, *Francisella tularensis*, and *Shigella* species genomic DNAs were obtained from the FDA facility and used for primer and probe specificity testing.

Food products. Shell eggs were purchased from local supermarkets and kept at 4°C until use.

Sample preparation. *Salmonella* strains were grown overnight on LB agar plates at 37°C. *Salmonella* strains from LB plates were inoculated into 50 ml of LB broth and grown standing overnight at 37°C. Cultures were harvested by centrifugation at 16,000 × *g* for 10 min at room temperature and washed once with phosphate-buffered saline (PBS), pH 7.4. For the preparation of artificially contaminated shell eggs, the cell suspension of the pure culture was added to shell eggs at a final population density of 10^8 CFU/ml. These cell suspensions were then blended for 5 s and 10-fold serially diluted in preblended raw shell eggs to obtain a range of samples containing approximately 10^7 to 10^1 CFU/ml. Uninoculated samples of shell eggs were also prepared as negative controls. A final volume of 2 ml of the corresponding sample dilutions was used immediately for infection of RAW 264.7 cell monolayers. Appropriate dilutions of salmonellae cells in the raw shell eggs were spread plated onto XLD agar and grown for at least 24 h in a 37°C incubator to determine accurate amounts of the initial inocula.

Tissue culture infections. RAW 264.7 macrophages (no. TIB-71; ATCC, Manassas, VA) were routinely grown at 37°C and 5% CO_2 in macrophage growth medium (Dulbecco's modified Eagle's medium [ATCC, Manassas, VA] containing 10% [vol/vol] fetal calf serum) supplemented with 100 μg/ml penicillin and streptomycin. RAW 264.7 macrophages were seeded into six-well tissue culture plates containing 2 ml of macrophage growth medium per well at a density of 1.5×10^6 cells per well and cultured to confluence for 24 h prior to use. Prior to infection, cultured cells were washed once with PBS. In two replicate experiments, cell monolayers were infected in duplicate with *Salmonella* in shell eggs or macrophage growth medium at population densities of 10^7 to 10^1 CFU/ml. Uninoculated shell eggs and macrophage growth medium were also added as negative controls. Plates were centrifuged at 600 × *g* for 5 min to promote bacterium-macrophage cell contact and incubated for 2 h at 37°C in 5% CO_2. Infected cell monolayers were then washed three times with PBS and reconstituted with macrophage growth medium alone and incubated for 5 h (postinfection) at 37°C in 5% CO_2. All macrophage monolayers were then transferred to microcentrifuge tubes and centrifuged at 600 × *g* for 10 min. Supernatants were removed, and macrophages were washed once with PBS. Macrophages were then resuspended in 30 μl of distilled H_2O. Macrophages were then boiled for 10 min, cooled for 5 min, and centrifuged at 16,000 × *g* for 10 min. The supernatants were used for RT-PCR analysis.

RT-PCR. All reactions were run at a final volume of 25 μl using the SmartCycler II apparatus (Cepheid, Sunnyvale, CA). The primers SEF14-F and SEF14-R and probe SEF14-P were previously designed and shown to specifically target a sequence found on the *Salmonella* serotype Enteritidis gene *sefA* (37). This fluorogenic probe contains a 5′ Cy3 fluorophore and a 3′ black hole quencher. The primers ORGC-F and ORGC-R and probe ORGC-P were designed to specifically target a sequence found on the *Salmonella* gene *orgC* (Table 1). That fluorogenic probe contains a 5′ Cy5 fluorophore and a 3′ black hole quencher. The reaction components and final concentrations used were 6 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 1 U of Takara Ex *Taq* Hot Start polymerase (Takara Bio, Inc., Madison, WI), 1× Ex *Taq* buffer, and 3 μl of each template DNA (primer and probe concentrations are shown in Table 1). Thermal cycling parameters were set for an initial 90-s denaturation step at 94°C, followed by 45 cycles at 94°C for 10 s for DNA denaturation with subsequent annealing and extension at 60°C for 15 s. The *orgC* and *sefA* RT-PCR analyses were run independently, and positive results were recorded as the measurement of at least 30 fluorescent units above the baseline occurring before the completion of 45 cycles.

Confirmatory identification. Shell eggs and macrophage growth medium contaminated with *Salmonella* serotype Enteritidis were prepared, and RAW 264.7 cell monolayers were infected as described above. At 5 h postinfection, RAW 264.7 cells were washed once with PBS and lysed by the addition of 80 μl of 0.1% sodium deoxycholate in PBS for approximately 2 min. The released bacteria were serial diluted 10-fold in PBS, plated onto XLD agar plates, and incubated at 37°C for at least 24 h or until the appearance of suspected *Salmonella* colonies. Suspected *Salmonella* colonies were streaked onto fresh XLD plates and incubated for at least 24 h at 37°C. Colonies were isolated and suspended in 30 μl of distilled H_2O in microcentrifuge tubes, boiled for 10 min, and centrifuged for 10 min, and 3 μl of the supernatant was used for RT-PCR identification with the *sefA*- and *orgC*-specific primers and probes. Positive confirmatory reactions were recorded as the measurement of at least 30 fluorescent units above the baseline occurring before the completion of 45 cycles.

Traditional culture method for detection of *Salmonella* phage types. The macrophage cell culture method for detection of *Salmonella* serotype Enteritidis phage types was run in parallel with the standard microbiological method performed according to the International Organization for Standardization (2).

TABLE 2. *Salmonella enterica* serotypes used to determine the specificity of the *orgC* and *sefA* primer and probe set

Salmonella enterica serotype or subspecies	No. of strains tested	RT-PCR result for indicated primer/probe set[a]	
		orgC	*sefA*
Enteritidis	16	+	+
Typhimurium	8	+	−
Java	1	+	−
Anatum	1	+	−
Newington	1	+	−
Newport	1	+	−
Infantis	1	+	−
Arizonae	1	+	−
Pullorum	1	+	−
Gallinarum	1	+	−
Poona	1	+	−
Chester	1	+	−
Havana	1	+	−
Derby	1	+	−
Johannesburg	1	+	−
Ohio	1	+	−
Braenderup	1	+	−
Tennessee	1	+	−
Istanbul	1	+	−
Cerro	1	+	−
Thompson	1	+	−
Worthington	1	+	−
Schwarzengrund	1	+	−
Agona	1	+	−
Senftenberg	1	+	−
Mbandaka	1	+	−
Montevideo	1	+	−
Hadar	1	+	−
Kentucky	1	+	−
Heidelberg	1	+	−
Saintpaul	1	+	−
Virchow	1	+	−
Choleraesuis	1	+	−

[a] +, positive PCR result; −, negative PCR result.

Briefly, spiked egg samples were inoculated into buffered peptone water (BPW) and grown for 18 h at 37°C. A 0.1-ml sample of growth in BPW was inoculated into 10 ml of Rappaport-Vassiliadis soya peptone broth and grown for 24 h at 41.5°C. A 1-ml sample of growth in BPW was also inoculated into 10 ml of tetrathionate broth and grown for 24 h at 37°C. Samples of *Salmonella* colony growth from Rappaport-Vassiliadis soya peptone and tetrathionate broth were streaked onto XLD and brilliant green agar plates and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies from XLD and brilliant green agar plates were subcultured onto tryptic soy agar (TSA) and incubated for 24 h at 37°C. Confirmation of *Salmonella* colonies from tryptic soy agar plates was achieved by using RT-PCR with the *sefA* primer and probe set.

RESULTS

RT-PCR primer and probe set specificity. The *sefA* primer and probe set (Table 1) was previously developed and shown to be specific for *Salmonella* serotype Enteritidis strains (37). The *sefA* gene encodes a subunit of the fimbrial antigen SEF14, which is essential for *Salmonella* cell binding to macrophages (11). The DNA sequence encoding SefA is unique to serogroup D members of salmonellae, including *Salmonella* serotype Enteritidis, and is absent from other *Salmonella* strains, including *Salmonella* serotype Typhimurium and non-*Salmonella* sp. food-borne bacterial pathogens (37). We confirmed that the *sefA* primer and probe set detected *Salmonella* serotype Enteritidis and displayed no cross-reactivity with other, non-serogroup D salmonellae, including *Salmonella* serotype Typhimurium (Table 2), or to a host of other potential food-borne bacterial pathogens (Table 3).

TABLE 3. *Salmonella* and non-*Salmonella* strains used to determine the specificity of the *orgC* and *sefA* primer and probe set

Organism	No. of strains tested	RT-PCR result for indicated primer/probe set[a]	
		orgC	*sefA*
Salmonella serotype Typhimurium	1	+	−
Salmonella serotype Enteritidis	1	+	+
Yersinia pestis	2	−	−
Yersinia enterocolitica	4	−	−
Yersinia pseudotuberculosis	4	−	−
Francisella tularensis subsp. *holarctica*	1	−	−
Francisella novicida	1	−	−
Listeria monocytogenes	1	−	−
Vibrio parahaemolyticus	9	−	−
Vibrio cholera	5	−	−
Enterobacter sakazakii	9	−	−
Clostridium botulinum	4	−	−
Shigella dysenteriae	1	−	−
Shigella sonnei	1	−	−
Shigella boydii	1	−	−
Escherichia coli O157:H7	1	−	−

[a] +, positive PCR result; −, negative PCR result.

The primer and probe set specific for *orgC* (Table 1) was designed to detect DNA from both serogroup D and non-serogroup D salmonellae, including *Salmonella* serotype Enteritidis and *Salmonella* serotype Typhimurium, respectively. The *orgC* gene is located on SPI-1 of the *Salmonella* chromosome and encodes a regulatory protein that is exported via a type III secretion system (10). Homology searches in the database have failed to uncover sequence similarities to the *orgC* gene, and it appears to encode a protein product unique to *Salmonella* species. In order to determine the *orgC* primer and probe set specificity, we conducted RT-PCR analysis of genomic DNA from several *Salmonella* serotype Enteritidis phage types, non-serogroup D salmonellae, and other potential food-borne bacterial pathogens. No fluorescent signal above background levels was observed after 45 cycles in any of the nonsalmonellae tested (Table 3), whereas all *Salmonella* serotypes tested showed fluorescent signals above background levels before the completion of 45 cycles (Table 2). These data indicate that the *orgC* primer and probe set is specific for salmonellae and does not cross-react with DNA from other potential food-borne bacteria.

Eukaryotic cell culture and RT-PCR identification of *Salmonella* serotype Enteritidis from artificially contaminated macrophage growth medium. *Salmonella* serotype Enteritidis has previously been shown to replicate within cultured macrophage cell lines in tissue culture dishes (7). In order to test the eukaryotic cell culture method, we used RAW 264.7 murine macrophages as the eukaryotic cell host. In two separate experiments performed in duplicate, RAW 264.7 macrophages were infected with *Salmonella* serotype Enteritidis phage type 13 in quantities ranging from 10^7 to 10^0 CFU/ml in macrophage growth medium. Analysis of the DNA from infected RAW 264.7 cell

TABLE 4. Mean cycle threshold values of *Salmonella* serotype Enteritidis (SE13) detection in macrophage medium by cell culture and RT-PCR analysis at 5 h postinfection using *orgC* and *sefA* primer and probe sets

Salmonella serotype Enteritidis amt (CFU/ml)	Cycle threshold value for indicated primer/probe set[a]	
	orgC	*sefA*
10^7	17.23 ± 0.47	19.41 ± 0.33
10^6	18.73 ± 0.60	21.28 ± 0.32
10^5	19.68 ± 0.80	22.59 ± 0.27
10^4	22.60 ± 0.26	25.16 ± 0.34
10^3	27.41 ± 0.54	30.56 ± 0.21
10^2	31.38 ± 0.31	34.78 ± 0.19
10^1	34.97 ± 0.32	38.17 ± 1.01
0	ND	ND

[a] Cycle threshold values are expressed as the means of two replicate experiments performed in duplicate ± standard deviations. ND, not detected.

lysates by RT-PCR revealed the detection limit of the assay to be 10 CFU/ml using the *orgC*- and *sefA*-specific primer and probe sets (Table 4). Lysates from RAW 264.7 cells exposed to uninoculated macrophage growth medium (negative control) failed to illicit a fluorescent signal before the completion of 45 cycles, indicating that the RT-PCR was specific for phage type 13 DNA and not for any nonspecific DNA sequence from the RAW 264.7 cell genome. Gel electrophoresis of the RT-PCR amplifications revealed DNA fragments with sizes corresponding to the *orgC* (121 bp) and *sefA* (98 bp) DNA segments, indicating that the fluorescent signal was derived specifically from phage type 13 DNA (data not shown). These data suggest that *Salmonella* serotype Enteritidis phage type 13 was internalized into RAW 264.7 cells and can subsequently be detected by RT-PCR from infected macrophage cell lysates.

Eukaryotic cell culture and RT-PCR detection of *Salmonella* serotype Enteritidis from artificially contaminated raw shell eggs. To evaluate the effectiveness of *Salmonella* serotype Enteritidis detection in raw shell eggs by using the eukaryotic cell culture technique and RT-PCR, RAW 264.7 macrophages were infected separately with *Salmonella* serotype Enteritidis phage types 4, 8, and 13 in quantities ranging from 10^7 to 10^1 CFU/ml in raw shell eggs (see Materials and Methods). Analysis of RAW 264.7 cell lysates from 5 h postinfection by RT-PCR revealed a detection limit of 10 CFU/ml for all three phage types when the *orgC*- and *sefA*-specific primer and probe sets were used (Table 5), which was identical to results obtained using the traditional culture method of the International Organization for Standardization (2). Lysates from RAW 264.7 cells exposed to uninoculated raw shell eggs (negative controls) failed to illicit a fluorescent signal before the completion of 45 cycles, indicating that the RT-PCR analyses were specific for *Salmonella* serotype Enteritidis DNA and not for nonspecific reactions with RAW 264.7 cell DNA or residual shell egg particles in the PCR mixture. These data demonstrate that the eukaryotic cell culture method and RT-PCR combination can correctly identify *Salmonella* serotype Enteritidis in raw shell eggs without a loss of specificity or sensitivity.

Differentiation between serogroup D and non-serogroup D salmonellae in artificially inoculated macrophage growth medium and raw shell eggs by eukaryotic cell culture and RT-PCR. The *orgC*-specific primer and probe set was designed to identify all *Salmonella* serotypes, including the two most common *Salmonella* serotypes implicated in human gastroenteritis, *Salmonella* serotype Enteritidis and *Salmonella* serotype Typhimurium. Conversely, the *sefA* primer and probe set detected a narrow range of *Salmonella* serotypes belonging to serogroup D, including *Salmonella* serotype Enteritidis. To determine whether the cell culture and RT-PCR method could be used to discriminate between serogroup D and non-serogroup D salmonellae, raw shell eggs were artificially inoculated with *Salmonella* serotype Typhimurium, a non-group D serotype, in quantities ranging from 10^7 to 10^1 CFU/ml, and macrophages were infected in two separate experiments performed in duplicate. Analysis of macrophage cell lysates from 5 h postinfection by RT-PCR analysis using the *orgC*-specific primer and probe set revealed a detection limit of 10 CFU/ml (Table 5). In contrast, no fluorescent signal was detected from macrophage cell lysates when the *sefA*-specific primer and probe set was used (Table 5). These data indicate that the cell culture and RT-PCR technique using the *orgC* and *sefA* primer and probe sets is a practical tool for the detection and differentiation of serogroup D (*orgC*$^+$ and *sefA*$^+$) and non-serogroup D (*orgC*$^+$ and *sefA* null) salmonellae.

Confirmatory identification of *Salmonella* serotype Enteritidis in raw shell eggs by the eukaryotic cell culture technique. A presumptive positive result by the eukaryotic cell culture and

TABLE 5. Mean cycle threshold values of *Salmonella* serotype Enteritidis (phage types 4, 8, and 13) and *Salmonella* serotype Typhimurium (phage type DT104) detection from artificially contaminated shell eggs by cell culture and RT-PCR at 5 h postinfection by using *orgC* and *sefA* primer and probe sets

Salmonella serotype amt (CFU/ml)	Cycle threshold value for indicated primer/probe set[a]							
	orgC				*sefA*			
	SE4	SE8	SE13	DT104	SE4	SE8	SE13	DT104
10^7	18.68 ± 0.17	18.13 ± 0.19	17.15 ± 0.58	16.41 ± 0.28	18.61 ± 0.57	18.14 ± 0.26	18.74 ± 0.75	ND
10^6	19.55 ± 0.73	19.56 ± 0.75	18.16 ± 0.78	17.36 ± 0.38	20.75 ± 0.96	20.83 ± 0.44	20.20 ± 0.35	ND
10^5	22.52 ± 0.41	22.14 ± 0.81	22.88 ± 1.39	20.70 ± 0.17	24.10 ± 0.46	23.51 ± 0.53	24.78 ± 0.51	ND
10^4	23.19 ± 0.16	26.69 ± 0.85	26.38 ± 0.51	24.40 ± 0.10	24.75 ± 0.19	28.07 ± 0.37	27.92 ± 1.17	ND
10^3	26.74 ± 1.11	29.18 ± 0.41	29.65 ± 0.72	27.72 ± 0.07	28.33 ± 1.44	30.49 ± 0.16	30.72 ± 1.44	ND
10^2	31.15 ± 1.01	30.93 ± 1.15	32.86 ± 2.05	31.80 ± 0.33	32.31 ± 0.97	32.79 ± 0.78	34.48 ± 4.01	ND
10^1	32.50 ± 2.06	34.91 ± 1.77	36.26 ± 2.55	40.46 ± 0.94	34.06 ± 0.86	34.68 ± 0.72	36.26 ± 3.52	ND
0	ND	ND	ND	ND	ND	ND	ND	ND

[a] Cycle threshold values are expressed as the means of two replicate experiments performed in duplicate ± standard deviations. ND, not detected.

RT-PCR method must be followed up by confirming the identity of *Salmonella* in pure culture. To evaluate the eukaryotic cell culture method for confirmatory identification, RAW 264.7 macrophages were infected with *Salmonella* serotype Enteritidis phage types 4, 8, and 13 in quantities ranging from 10^7 to 10^0 CFU/ml in raw shell eggs. At 5 h postinfection, macrophages were lysed with 0.1% sodium deoxycholate in PBS, and the released salmonellae were plated on XLD agar and incubated for at least 24 h at 37°C. Suspected colonies were streaked for isolation on XLD agar and incubated for at least 24 h. Colonies were chosen and boiled as described in Materials and Methods, and the released DNA was analyzed by RT-PCR using the *sefA*- and *orgC*-specific primer and probe sets. Confirmatory identification revealed a detection limit of 10 CFU/ml (data not shown). Lysates from RAW 264.7 cells exposed to uninoculated raw shell eggs (negative control) failed to produce *Salmonella* serotype Enteritidis colonies on XLD agar plates.

DISCUSSION

Methods used previously to detect *Salmonella* from food specimens include classic microbiologically based (cultivation, biochemical profiling) and immunologically based (enzyme-linked immunosorbent assay, direct fluorescence antibody) methods (25, 28, 29, 30, 39). These methods are frequently being replaced with more-rapid and -sensitive molecular biology-based techniques. RT-PCR is a recent advance in molecular detection that has been used successfully for the rapid identification of a wide array of bacterial pathogens in specific food matrices (12, 15, 22, 34, 36, 40). However, various food products have been shown to interfere with PCR assays, resulting in potentially false-negative results (1, 22, 31, 32, 33, 35). Indeed, detection of *Salmonella* in raw shell eggs by RT-PCR has been challenging due to the PCR-inhibitory nature of eggs (41). Therefore, a sample processing step is often required to isolate the target organism from potential PCR-inhibitory food particles prior to initiating the RT-PCR assay. Preenrichment strategies using selective broth have been employed prior to the application of RT-PCR for detection of *Salmonella* in eggs in order to dilute the food matrix and increase the bacterial load to detectable levels (26, 37). However, the total time for detection of *Salmonella* species using these preenrichment steps has exceeded 24 h in each case.

We sought to develop a system for the specific identification of serogroup D salmonellae, which include *Salmonella* serotype Enteritidis, in raw shell eggs that can (i) isolate or separate *Salmonella* serotype Enteritidis from PCR-inhibitory food particles, (ii) enrich small amounts of *Salmonella* serotype Enteritidis to detectable levels for PCR, (iii) detect only viable cells, and (iv) isolate *Salmonella* serotype Enteritidis colonies for subsequent confirmatory identification and that is rapid, sensitive, and specific. The technique presented here uses a macrophage cell monolayer that acts as host for the physical separation of *Salmonella* from egg particles. Once *Salmonella* cells are separated from inhibitory egg components and concentrated via intracellular multiplication to detectable levels, the bacterium can be identified via RT-PCR. We show that this system is capable of identifying as little as 10 CFU/ml of *Salmonella* serotype Enteritidis and *Salmonella* serotype Typhimurium in raw eggs and that it displays a broad range of detection, from 10^7 CFU/ml to 10^1 CFU/ml. The overall analysis time for presumptive detection of *Salmonella* in raw eggs was 10 h, with no false positives recorded. Another advantage of this eukaryotic cell culture technique is that only living *Salmonella* cells are able to reside and multiply within macrophages, thus restricting detection to viable bacteria. Furthermore, a modification of the procedure allows confirmatory detection of *Salmonella* serotype Enteritidis in eggs in less than 48 h, compared to the more than 5 days required for the standard method of the International Organization for Standardization (2).

Primer and probe sets that recognize DNA sequences unique to and present in all *Salmonella* serotype Enteritidis strains have yet to be developed. The *Salmonella sefA* and Prot6e genes have been used previously as targets for the RT-PCR identification of *Salmonella* serotype Enteritidis strains in raw eggs and have come the closest to satisfying inclusivity and exclusivity criteria (26, 37). The *sefA* DNA sequence appears to be present in all known *Salmonella* serotype Enteritidis phage types tested so far but is not unique to *Salmonella* serotype Enteritidis and is present in seven other *Salmonella* serotypes, all of which are members of serogroup D (37). In contrast, Prot6e is found exclusively in *Salmonella* serotype Enteritidis strains and is absent from all other known *Salmonella* serotypes, including other members of serogroup D (26). However, Prot6e is carried on a plasmid, which is prone to loss or modification (26). Therefore, *Salmonella* serotype Enteritidis strains lacking a virulence plasmid would not be detected using Prot6e primers and probes, leading to false-negative RT-PCR assays. In order to avoid potentially false-negative results, we chose *sefA* as the RT-PCR target gene. We also utilized a primer and probe set specific for the *Salmonella* gene *orgC* in order to add a degree of specificity and to distinguish between serogroup D and non-serogroup D salmonellae when used in combination with the *sefA* primer and probe set. We showed that the *orgC*-specific primer and probe set recognizes DNA from 55 *Salmonella* strains representing 33 serotypes, including 16 strains of *Salmonella* serotype Enteritidis and 8 strains of *Salmonella* serotype Typhimurium (Table 2). Moreover, the *orgC* primer and probe set exhibited no cross-reactivity to DNA from 46 strains representing 14 potential bacterial food-borne pathogens (Table 3). Using the combination of these two primer/probe sets allows us to determine the presence of serogroup D members, including *Salmonella* serotype Enteritidis (*orgC*$^+$ and *sefA*$^+$), non-serogroup D *Salmonella* serotypes (*orgC*$^+$ and *sefA* null), or no *Salmonella* contamination (*orgC* null and *sefA* null). Although *Salmonella* serotype Enteritidis is the predominant cause of food poisoning among members of serogroup D, the current primers and probes available do not allow us to definitively verify the presence of *Salmonella* serotype Enteritidis in a particular food outbreak. The discovery of a unique chromosomal DNA sequence present in all *Salmonella* serotype Enteritidis serotypes would allow us to combine the RT-PCR procedure with the eukaryotic cell culture isolation technique to rapidly substantiate the presence of *Salmonella* serotype Enteritidis in raw eggs. However, this unique sequence is unlikely to be found on the invasion-associated pathogenicity island SPI-1, which is a region of the *Salmonella* chromosome that is commonly used

for identification by PCR, in light of the recent discovery of human clinical isolates of *Salmonella* serotype Senftenberg that do not possess SPI-1 (21).

In theory, the eukaryotic cell culture and RT-PCR technique, or variations of this technique, could be used to detect other food-borne pathogens that exhibit intracellular life cycles, such as *Listeria monocytogenes*, *Shigella* spp., and *Yersinia* spp. In fact, previous experiments have demonstrated that a modified version of the eukaryotic cell culture and RT-PCR method can identify as few as 10 CFU/ml of *Francisella tularensis* in both solid and liquid food matrices (J. B. Day, unpublished data). The method may also conceivably be employed for the detection of salmonellae in water as well as in environmental and clinical samples. Although the method presented here was designed for the rapid analysis of small egg samples that are suspected of harboring *Salmonella* serotype Enteritidis or other *Salmonella* serotypes, the technique can conceivably be adapted for high-throughput identification of *Salmonella* spp. in larger samples by using automated microtiter plate assay formats with subsequent detection by RT-PCR. However, the requirement for macrophage cell lines to perform the detection method may restrict the application of this technique to laboratories that have access to tissue culture facilities and personnel skilled in cell culture techniques.

The pandemic nature of *Salmonella* species throughout the world and the potential severity of infection from ingestion have raised awareness of the importance of developing rapid and sensitive techniques to detect *Salmonella* in food matrices, particularly egg products. The method described here would allow the identification of *Salmonella* species within 10 h so that further exposure to the public could be prevented and the timely administration of appropriate antibiotics could be initiated in the event of an outbreak. In addition, confirmation could be achieved within 48 h without the need for prolonged incubation in selective enrichment media.

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