

3-2013


# Detection of Salmonella Enteritidis in Pooled Poultry Environmental Samples Using a Serotype-Specific Real-Time–Polymerase Chain Reaction Assay

Derek R. Adams  
*Iowa State University*

Wendy R. Stensland  
*Iowa State University, wbuff@iastate.edu*

Chong Wang  
*Iowa State University, chwang@iastate.edu*

Annette O'Connor  
Follow this and additional works at: [http://lib.dr.iastate.edu/vdpam\\_pubs](http://lib.dr.iastate.edu/vdpam_pubs)  
*Iowa State University, oconnor@iastate.edu*

 Part of the [Large or Food Animal and Equine Medicine Commons](#), [Other Veterinary Medicine Commons](#), [Veterinary Infectious Diseases Commons](#), and the [Veterinary Pathology and Pathobiology Commons](#)  
*Iowa State University*

*See next page for additional authors.*  
The complete bibliographic information for this item can be found at [http://lib.dr.iastate.edu/vdpam\\_pubs/66](http://lib.dr.iastate.edu/vdpam_pubs/66). For information on how to cite this item, please visit <http://lib.dr.iastate.edu/howtocite.html>.

---

# Detection of *Salmonella* Enteritidis in Pooled Poultry Environmental Samples Using a Serotype-Specific Real-Time–Polymerase Chain Reaction Assay

## Abstract

While real-time–polymerase chain reaction (RT PCR) has been used as a rapid test for detection of *Salmonella* Enteritidis in recent years, little research has been done to assess the feasibility of pooling poultry environmental samples with a *Salmonella* Enteritidis–specific RT PCR assay. Therefore the objective of this study was to compare RT PCR *Salmonella* Enteritidis detection in individual and pooled (in groups of two, three, and four) poultry environmental drag swab samples to traditional cultural methods. The drag swabs were collected from poultry facilities previously confirmed positive for *Salmonella* Enteritidis and were cultured according to National Poultry Improvement Plan guidelines. Initial, *Salmonella* Enteritidis–specific RT PCR assay threshold cycle cutoff values of  $\leq 36$ ,  $\leq 30$ , and  $\leq 28$  were evaluated in comparison to culture. The average limit of detection of the RT PCR assay was  $2.4 \times 10^3$  colony-forming units (CFUs)/ml, which corresponded to an average threshold cycle value of 36.6. Before enrichment, samples inoculated with concentrations from  $10^2$  to  $10^5$  CFUs/ml were detected by RT PCR, while after enrichment, samples inoculated from  $10^0$  to  $10^5$  CFUs/ml were detected by RT PCR. Threshold cycle cutoff values were used in the subsequent field trial from which *Salmonella* Enteritidis was cultured in 7 of 208 environmental samples (3.4%). Individual samples were 99.0%, 100%, and 100% in agreement with the RT PCR at threshold cycle (*Ct*) cutoff values of  $\leq 36$ ,  $\leq 30$ , and  $\leq 28$  respectively. The agreement for pooled samples also followed the same trend with highest agreement at  $Ct \leq 28$  (pool of 2 = 100.0%, pool of 3 = 100.0%, pool of 4 = 100.0%), midrange agreement at  $Ct \leq 30$  (pool of 2 = 99.0%, pool of 3 = 100.0%, pool of 4 = 100.0%), and lowest agreement at  $Ct \leq 36$  (pool of 2 = 98.1%, pool of 3 = 97.1%, pool of 4 = 98.1%). In conclusion, regardless of the level of pooling after tetrathionate enrichment, sensitivity was very good, and results would be comparable to what would have been found with individual culture or individual RT PCR at  $Ct \leq 36$ .

## Keywords

poultry, drag swab, detection, pooled, real-time polymerase chain reaction, *Salmonella* Enteritidis

## Disciplines

Large or Food Animal and Equine Medicine | Other Veterinary Medicine | Veterinary Infectious Diseases | Veterinary Pathology and Pathobiology

## Comments

This article is from *Avian Diseases* 57 (2013); 22, doi: [10.1637/10279-061312-Reg.1](https://doi.org/10.1637/10279-061312-Reg.1). Posted with permission.

## Authors

Derek R. Adams, Wendy R. Stensland, Chong Wang, Annette O'Connor, Darrell W. Trampel, Karen M. Harmon, Erin L. Strait, and Timothy S. Frana

## **Detection of *Salmonella* Enteritidis in Pooled Poultry Environmental Samples Using a Serotype-Specific Real-Time–Polymerase Chain Reaction Assay**

Author(s): Derek R. Adams, Wendy R. Stensland, Chong H. Wang, Annette M. O'Connor, Darrell W. Trampel, Karen M. Harmon, Erin L. Strait, and Timothy S. Frana

Source: *Avian Diseases*, 57(1):22-28.

Published By: American Association of Avian Pathologists

DOI: <http://dx.doi.org/10.1637/10279-061312-Reg.1>

URL: <http://www.bioone.org/doi/full/10.1637/10279-061312-Reg.1>

---

BioOne ([www.bioone.org](http://www.bioone.org)) is a nonprofit, online aggregation of core research in the biological, ecological, and environmental sciences. BioOne provides a sustainable online platform for over 170 journals and books published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Web site, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at [www.bioone.org/page/terms\\_of\\_use](http://www.bioone.org/page/terms_of_use).

Usage of BioOne content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

## Detection of *Salmonella* Enteritidis in Pooled Poultry Environmental Samples Using a Serotype-Specific Real-Time–Polymerase Chain Reaction Assay

Derek R. Adams, Wendy R. Stensland, Chong H. Wang, Annette M. O'Connor, Darrell W. Trampel, Karen M. Harmon, Erin L. Strait, and Timothy S. Frana<sup>A</sup>

Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011

Received 14 June 2012; Accepted 25 September 2012; Published ahead of print 26 September 2012

**SUMMARY.** While real-time–polymerase chain reaction (RT PCR) has been used as a rapid test for detection of *Salmonella* Enteritidis in recent years, little research has been done to assess the feasibility of pooling poultry environmental samples with a *Salmonella* Enteritidis–specific RT PCR assay. Therefore the objective of this study was to compare RT PCR *Salmonella* Enteritidis detection in individual and pooled (in groups of two, three, and four) poultry environmental drag swab samples to traditional cultural methods. The drag swabs were collected from poultry facilities previously confirmed positive for *Salmonella* Enteritidis and were cultured according to National Poultry Improvement Plan guidelines. Initial, *Salmonella* Enteritidis–specific RT PCR assay threshold cycle cutoff values of  $\leq 36$ ,  $\leq 30$ , and  $\leq 28$  were evaluated in comparison to culture. The average limit of detection of the RT PCR assay was  $2.4 \times 10^3$  colony-forming units (CFUs)/ml, which corresponded to an average threshold cycle value of 36.6. Before enrichment, samples inoculated with concentrations from  $10^2$  to  $10^5$  CFUs/ml were detected by RT PCR, while after enrichment, samples inoculated from  $10^0$  to  $10^5$  CFUs/ml were detected by RT PCR. Threshold cycle cutoff values were used in the subsequent field trial from which *Salmonella* Enteritidis was cultured in 7 of 208 environmental samples (3.4%). Individual samples were 99.0%, 100%, and 100% in agreement with the RT PCR at threshold cycle ( $C_t$ ) cutoff values of  $\leq 36$ ,  $\leq 30$ , and  $\leq 28$  respectively. The agreement for pooled samples also followed the same trend with highest agreement at  $C_t \leq 28$  (pool of 2 = 100.0%, pool of 3 = 100.0%, pool of 4 = 100.0%), midrange agreement at  $C_t \leq 30$  (pool of 2 = 99.0%, pool of 3 = 100.0%, pool of 4 = 100.0%), and lowest agreement at  $C_t \leq 36$  (pool of 2 = 98.1%, pool of 3 = 97.1%, pool of 4 = 98.1%). In conclusion, regardless of the level of pooling after tetrathionate enrichment, sensitivity was very good, and results would be comparable to what would have been found with individual culture or individual RT PCR at  $C_t \leq 36$ .

**RESUMEN.** Detección de *Salmonella* Enteritidis en muestras ambientales avícolas agrupadas, utilizando un ensayo de reacción en cadena de la polimerasa en tiempo real específico de serotipo.

No obstante que en los años recientes el método de reacción en cadena de la polimerasa en tiempo real (RT-PCR) ha sido utilizado como una prueba rápida para la detección de *Salmonella* Enteritidis, se ha realizado poca investigación para evaluar la viabilidad de agrupar las muestras ambientales avícolas para realizar un ensayo específico de RT-PCR para *Salmonella* Enteritidis. Por lo tanto, el objetivo de este estudio fue comparar el método de RT-PCR para la detección de *Salmonella* Enteritidis con muestras de hisopos de arrastre de instalaciones avícolas individuales y agrupadas (en grupos de dos, tres o cuatro hisopos) con los métodos de cultivo tradicionales. Los hisopos se obtuvieron de instalaciones avícolas que fueron previamente confirmadas positivas para *Salmonella* Enteritidis y se cultivaron de acuerdo con las especificaciones del Plan Nacional para el Mejoramiento Avícola. Se evaluaron los valores iniciales de corte de los ciclos umbrales de  $\leq 36$ ,  $\leq 30$ , y  $28 \leq$  del método específico de RT-PCR para *S.* Enteritidis en comparación con el cultivo. El límite promedio de detección del ensayo de RT-PCR fue de  $2.4 \times 10^3$  unidades formadoras de colonias (UFC)/ml, lo cual corresponde a un valor promedio de ciclo umbral de 36.6. Las muestras inoculadas con concentraciones de  $10^2$  a  $10^5$  UFC/ml fueron detectadas por RT-PCR antes del enriquecimiento, mientras que las muestras inoculadas con concentraciones de  $10^0$  a  $10^5$  UFC/ml fueron detectadas por la RT PCR después del enriquecimiento. Los valores de corte de los ciclos umbrales se utilizaron en una prueba de campo posterior donde se cultivó *S.* Enteritidis en 7 de 208 muestras ambientales (3.4%). Las muestras individuales mostraron una concordancia de 99.0%, 100%, y 100% con los valores de corte de los ciclos umbrales ( $C_t$ ) de  $\leq 36$ ,  $\leq 30$ , y  $\leq 28$ , respectivamente. Las muestras agrupadas mostraron la misma tendencia con un mayor nivel de concordancia con los valores de  $C_t \leq 28$  (grupo de 2 = 100.0%, grupo de 3 = 100.0%, grupo de 4 = 100.0%), se observó una concordancia intermedia con un  $C_t \leq 30$  (grupo de 2 = 99.0%, grupo de 3 = 100.0%, grupo de 4 = 100.0%) y la concordancia más baja con un  $C_t \leq 36$  (grupo de 2 = 98.1%, grupo de 3 = 97.1%, grupo de 4 = 98.1%). En conclusión, independientemente del grado de agrupación después de enriquecimiento con caldo tetratiionato, la sensibilidad fue muy buena, y los resultados fueron comparables a lo que se habrían encontrado con un cultivo individual o con un procesamiento individual por RT-PCR con un  $C_t \leq 36$ .

**Key words:** poultry, drag swab, detection, pooled, real-time polymerase chain reaction, *Salmonella* Enteritidis

**Abbreviations:** BGN = brilliant green with novobiocin; CFUs/ml = colony-forming units per milliliter; FDA = Food and Drug Administration; MSR/V = modified semisolid Rappaport-Vassiliadis; MIL = motility-indole-lysine; NPIP = National Poultry Improvement Plan; NVSL = National Veterinary Services Laboratories; RT PCR = real-time polymerase chain reaction; SE = *Salmonella* Enteritidis;  $C_t$  = threshold cycle; TSI = triple sugar iron; TSA = trypticase soy agar; TG ROC = two-graph receiver operating characteristic; XLT4 = xylose-lysine-tergitol

<sup>A</sup>Corresponding author. E-mail: tfrana@iastate.edu

Disclaimer: The authors declare that they do not have any conflict of interest in their authorship and publication of this contribution. The authors received no financial support for the research, authorship, and/or publication of this article.

*Salmonella enterica* serovar Enteritidis (SE) has emerged in the past 30 years as a leading cause of human salmonellosis in the United States (3,15). In 2009 SE represented 19.2% of all laboratory confirmed *Salmonella* infections reported through FoodNet, surpassing all other serotypes (16). Between 1990 and 2001, 78% of SE outbreaks with a known source originated from shell eggs (10). Given the threat that SE presents to public health, new regulations enacted by the U.S. Food and Drug Administration (FDA) in 2010 made environmental testing mandatory for the majority of producers (10). If SE is isolated from the environment of chicken houses, then eggs from SE-positive houses must be tested. Testing eggs for SE requires a large sample size because only a small proportion of eggs are contaminated in an infected flock (11). Therefore, environmental sampling is the primary means by which flocks are monitored for SE. Environmental (or egg) testing has traditionally been carried out using bacterial culture, which is the standard by which all other tests are compared. Culture methods for environmental samples generally consist of pre-enrichment and selective enrichment steps followed by plating on selective media, biochemical testing, and serological testing of *Salmonella* colonies to confirm suspects (2,20). Bacteriological culturing typically requires 5 to 7 days before results are obtained. Real-time-polymerase chain reaction (RT PCR) is one testing method that has been developed to decrease the time required for testing. Coupled with an enrichment step, results are usually obtained in only 2 days (4,17). For the detection of *Salmonella*, most studies support the conclusion that the sensitivity and specificity of PCR matches or exceeds that of culture (5,8,13,19).

*Salmonella* testing using culture-based methods is a significant cost to producers associated with implementation of the FDA's Final Rule and is expected to cost producers an annual \$4.6 million in environmental testing and \$9.7 million in egg testing (10). Sample pooling is one strategy to reduce costs and labor associated with testing. Egg pooling has been shown to be effective in detecting SE when combined with RT PCR (17). However, little has been published considering the use of an SE-specific RT PCR assay for testing pooled poultry environmental swabs. A pooled, SE-specific RT PCR assay with its advantages of reduced cost, labor, and testing time over conventional culture presents a useful option for poultry environmental testing.

Objectives of this study were to 1) ascertain the limits of detection for an SE-specific RT PCR test, 2) determine the effect of enrichment media on sensitivity of the RT PCR test, and 3) compare detection of SE in individual and pooled poultry environmental drag swab samples using RT PCR. Pool sizes of two, three, and four were chosen to compare with individual sample testing and with culture.

## MATERIALS AND METHODS

To examine the validity of an SE-specific RT PCR in pooled samples, a study was performed that compared detection of SE in individual and pooled poultry environmental samples using the provisionally approved National Poultry Improvement Plan (NPIP) modified semisolid Rappaport-Vassiliadis (MSRV) method as the gold standard (1). RT PCR results from pool sizes of two, three, and four samples were compared with single-sample testing and with culture. The limit of detection for each pool size and effect of enrichment on detection were performed to assess performance of the SE RT PCR.

**Limit of detection study.** To determine the lower limit of detection of the RT PCR assay, a serial dilution study was carried out using three Group D *Salmonella* field isolates confirmed as SE by the National Veterinary Services Laboratories (NVSL), Ames, Iowa. A standard solution for each of the three SE isolates was prepared by inoculating

Table 1. Threshold cycle ( $C_t$ ) values for three *Salmonella* Enteritidis (SE) isolates serially diluted ( $3 \times 10^{-1}$  to  $10^8$ ) and tested using SE-specific RT PCR.

Average dilution concentration (CFU/ml)	Isolate 1	Isolate 2	Isolate 3	Average $C_t$
$3 \times 10^8$	17.43	17.34	17.22	17.33
$3 \times 10^7$	22.29	22.41	22.17	22.29
$3 \times 10^6$	25.54	26.20	24.82	25.52
$3 \times 10^5$	28.71	28.80	28.76	28.76
$3 \times 10^4$	31.70	32.69	31.42	31.94
$3 \times 10^3$	35.46	35.39	37.03	35.96
$3 \times 10^2$	37.30	—	—	37.30 <sup>A</sup>
$3 \times 10^1$	—	—	—	—
$3 \times 10^0$	—	—	—	—
$3 \times 10^{-1}$	—	—	—	—

<sup>A</sup>Only one isolate tested positive at  $10^2$  CFUs/ml. Therefore the  $C_t$  value does not represent the average of the three isolates.

overnight growth of organisms into saline to a 0.5 McFarland standard. Tenfold dilutions of the standard with an estimated range from  $10^{-1}$  to  $10^8$  CFUs/ml were prepared and 100  $\mu$ l spread plated onto sheep blood agar. Each dilution set was comprised of nine positive dilutions and one negative control. Dilutions were incubated at 37 C ( $\pm$  1 C) for 22 hr ( $\pm$  2 hr), and the number of colony-forming units per milliliter (CFUs/ml) for each dilution was calculated. An aliquot of each dilution was then submitted for RT PCR. The limit of detection of the RT PCR was calculated as the CFUs/ml of the lowest dilution at which a threshold cycle ( $C_t$ ) value was detected.

**Sensitivity of RT PCR assay with enrichment.** To assess the effect of tetrathionate enrichment on the sensitivity of the RT PCR, dilutions of the same three SE isolates described above were prepared in tenfold increments between  $10^0$  and  $10^5$  CFUs/ml. Two sets of dilutions containing comparable concentrations of SE were formed with each set comprising six positive dilutions and one negative control. A 1 ml aliquot of each dilution and negative control was used to inoculate a drag swab presoaked in sterilized skim milk. Drag swabs were transferred to Whirl-Pak bags (Nasco, Fort Atkinson, WI) with the first set containing no fecal material and the second set containing 5 g of SE-negative chicken feces. Then 100 ml tetrathionate was added to each bag. Aliquots of 1 ml were taken from each sample before and after incubation ( $37 \pm 1$  C/22  $\pm$  2 hr) and frozen at  $-20$  C. All aliquots were submitted for RT PCR analysis.

**Field study.** A total of 208 environmental field samples were collected from three commercial layer houses on the same site. Houses were previously found to be positive for SE by culture at the ISU VDL. Each house contained 12 rows of cages with three tiers of cages within each row. Flocks within each house consisted of adult laying hens. Gauze drag swabs presoaked with sterilized skim milk were used to sample egg belt sections from each tier of cages within each row and from fecal material on support beams directly under the cage section sampled. Samples were taken every 50 feet along the length of the house. Swabs were put into Whirl-Pak bags and transported on ice to the ISU VDL for testing.

**Bacterial culture.** A 100 ml tetrathionate solution was added to each Whirl-Pak bag and this enrichment broth incubated at 42 C ( $\pm$  1 C) for 22 hr ( $\pm$  2 hr). After incubation, 100  $\mu$ l of enriched tetrathionate solution was injected under the surface of MSRV media (Difco BD, Franklin Lakes, NJ). The media were incubated for 42 C ( $\pm$  1 C) for 22 hr ( $\pm$  2 hr) and observed for a halo of motility around the inoculation site. Positive suspects were plated onto brilliant green with novobiocin (BGN) agar and xylose-lysine-tergitol (XLT4) agar (both Difco) for further testing. If negative, samples were incubated for an additional 42 C ( $\pm$  1 C) for 22 hr ( $\pm$  2 hr) in the event the *Salmonella* in question was weakly motile. Regardless, if growth was present on the MSRV plate with or without motility at 48 hr, the sample was cultured further on BGN and XLT4 media.

Table 2. Average threshold cycle ( $C_t$ ) values of three trials for sample with diluted inoculations ( $\log 10^0$  to  $10^5$  CFUs/ml) tested using SE-specific RT PCR before and after enrichment with tetrathionate.

Average dilution concentration (CFU/ml)	Drag swab/no feces		Drag swab with feces	
	Before enrichment	After enrichment	Before enrichment	After enrichment
Log $10^5$	28.76	15.68	26.67	17.33
Log $10^4$	32.41	15.13	29.60	16.77
Log $10^3$	35.44	15.30	33.67	18.93
Log $10^2$	—	15.62	35.45 <sup>A</sup>	23.17
Log $10^1$	—	15.60	—	19.77
Log $10^0$	—	15.38	—	24.67
Negative control	—	—	—	—

<sup>A</sup>Only two of three samples tested positive at this concentration level. Therefore the average is only for those two isolates.

After plating onto BGN and XLT4, suspects were incubated at 37 C ( $\pm 1$  C) for 22 hr ( $\pm 2$  hr). From those plates, five suspects were selected per sample and were inoculated into triple sugar iron (TSI) and motility-indole-lysine (MIL) media (both Difco) and incubated at 37 C ( $\pm 1$  C) for 22 hr ( $\pm 2$  hr). Suspects were plated onto trypticase soy agar (TSA) with 5% sheep blood agar (Remel Products, Lenexa KS) and then serogrouped using an agglutination test using poly "O," poly "H" antisera (Difco), and O:9 antisera (Statens Serum Institut, Copenhagen, Denmark). All isolates testing positive for Group D were sent to NVSL for serotyping and SE confirmation.

**Sample pooling.** After incubation, 1 ml aliquots were removed from the enrichment broth of field environmental samples for RT PCR analysis. Sets of pooled samples were prepared from these aliquots so that each individual sample was represented once and randomly assigned to a pooled set of 2, 3, or 4 samples (208 individual, 104 pools of two, 70 pools of three, and 52 pools of four). Two uninoculated, negative samples were used to make 70 pools of three. Random allocation was determined using statistical software (SAS version 9.2; SAS Institute Inc., Cary, NC).

**Real-time polymerase chain reaction. DNA preparation.** DNA was purified, from enriched samples, using a commercially available DNA extraction kit (PrepSEQ<sup>®</sup> nucleic Acid Extraction; Applied Biosystems, Foster City, CA) and a magnetic particle processor (KingFisher 96; Thermo Electron Corporation, Hudson, NH).

**RT PCR.** A commercial kit (TaqMan<sup>®</sup> *Salmonella* Enteritidis Detection Kit; Applied Biosystems, Foster City, CA) was used to perform the real-time PCR.  $C_t$  cutoff values were not included in the protocol because the kit was considered a screen test with culture confirmation recommended for PCR-positive samples. The kit contains primers and a TaqMan<sup>®</sup> labeled probe (FAM<sup>™</sup> dye) that specifically target and amplify *Salmonella* Enteritidis. An internal positive control (VIC<sup>®</sup> dye) is also included to monitor assay validity and determine the presence of inhibition. Total reaction volume was 30  $\mu$ l, which consisted of 12  $\mu$ l of extracted DNA, 15  $\mu$ l of 2 $\times$  Environmental Master Mix 2.0, and 3  $\mu$ l *Salmonella* Enteritidis 10 $\times$  Assay Mix. PCR was performed in a real-time PCR instrument (ABI<sup>™</sup> 7500 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA) using the following program: 1 cycle of 10 min at 95 C, and 40 cycles of 15 sec at 95 C and 1 min at 60 C. Amplification plots were viewed and analyzed using the default settings in Sequence Detection Software version 1.7 (Applied Biosystems). A sample exhibiting a sigmoidal curve with a  $C_t$  before the end of the run in both the FA and VIC dye sets was considered positive.

**Analysis. Field study.** The total number of culture-positive and RT PCR-positive samples were determined. RT PCR results from field environmental samples that had threshold cycle values ( $C_t$ ) less than 40 were grouped by culture results (positive or negative) and the mean  $C_t$  values compared. A two-sample *t*-test was conducted using SAS

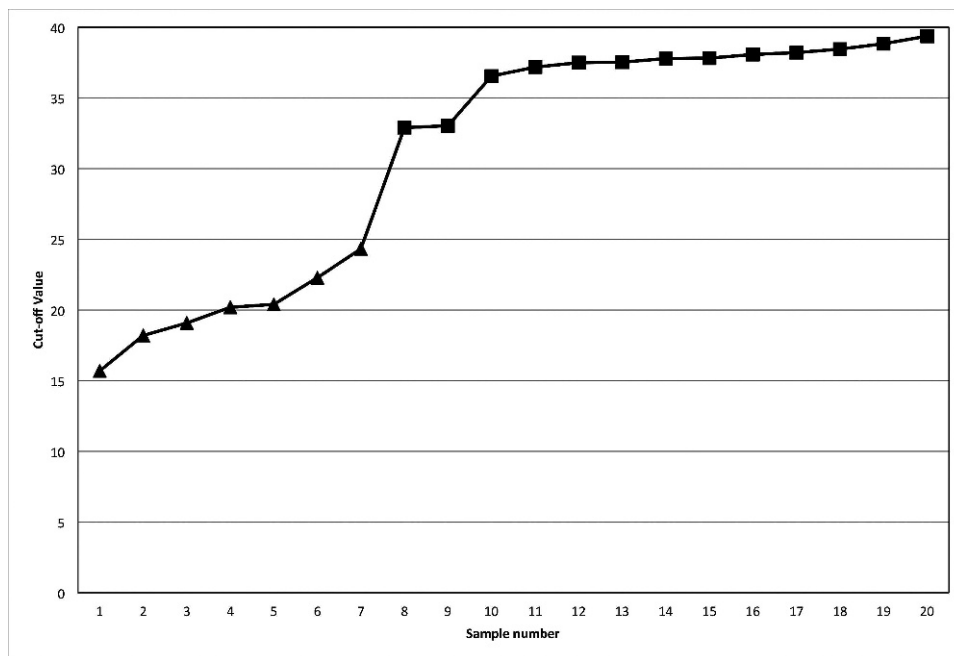


Fig. 1. Chart of RT PCR cutoff values from individual samples. Black triangles (▲) indicate that *Salmonella* Enteritidis was detected in the sample by culture. Black squares (■) indicate that *Salmonella* Enteritidis was not detected in the sample by culture.

statistical software on the mean  $C_t$  values for these groups to determine whether they were statistically different.

**Two-graph receiver operator characteristic plots.** A two-graph receiver operating characteristic (TG ROC) curve analysis was carried out using SAS statistical software to determine the optimal  $C_t$  cutoff for each of the groups (individual, pool of 2, pool of 3, pool of 4) using the RT PCR and culture results. One method of calculating the  $C_t$  cutoff is by using a TG ROC plot of diagnostic sensitivity *vs.* diagnostic specificity (7). On a TG ROC plot, diagnostic sensitivity and diagnostic specificity are plotted together on the same graph. The diagnostic sensitivity curve begins at 0% at a low  $C_t$  and increases toward 100% as the  $C_t$  cutoff is increased, while conversely the diagnostic specificity curve begins at 100% at a low  $C_t$  and decreases to 0% as the  $C_t$  cutoff is increased. From the graph, a  $C_t$  cutoff value can be selected that maximizes sensitivity or specificity or looks for the best combination of the two. When both are equally valued, the cutoff that gives equal sensitivity and specificity is selected. On a TG ROC plot, this is associated with the point at which the curves intersect. Setting a cutoff value resolves the uncertainty associated with high  $C_t$  values and ultimately improves the accuracy of the RT PCR assay.

**Agreement among cutoffs.** Analyzed results from limit of detection study, enrichment study, and TG ROC plots were used to select  $C_t$  cutoff values to test for agreement between RT PCR and culture results in each of the groups (individual, pool of two, pool of three, pool of four). Several  $C_t$  cutoff values of 36, 30, and 28 were tested for agreement to determine effect of different cutoff values on sensitivity and specificity. SAS statistical software was used to test agreement by calculating percentage agreement and kappa statistics (9). Percentage agreement was used to calculate the percentage of results that agreed between culture and individual/pooled RT PCR. Cohen's kappa was used to calculate the agreement between culture and individual/pooled RT PCR that removed the agreement expected by chance alone (9). A RT PCR-pooled sample was considered culture-positive if it had at least one culture-positive individual within the pool. In the same manner, pooled samples were considered RT PCR-positive if they generated a  $C_t$  value below the cutoff and had at least one RT PCR-positive individual within the pool.

## RESULTS

**Limit of detection study.** The limit of detection of the RT PCR assay ranged from  $2 \times 10^5$  to  $4 \times 10^3$  CFUs/ml (Table 1). When averaged, the limit of detection for the PCR assay was found to be  $2.4 \times 10^3$  CFUs/ml. The average  $C_t$  values for the three isolates across all dilutions ranged from 17.2 to detect a concentration of  $3 \times 10^8$  to 37.3 to detect  $3 \times 10^2$  SE CFUs/ml. The average  $C_t$  value for the lowest dilution at which a  $C_t$  was recorded across all isolates was 36.6.

**Sensitivity of RT PCR assay with enrichment.** Before enrichment, dilutions from spiked samples provided similar results as in the limit of detection study (Table 2). Fecal-negative samples inoculated with concentrations from  $10^3$  to  $10^5$  CFUs/ml were detected by RT PCR with mean  $C_t$  values of 28.76 ( $3 \times 10^5$  CFUs/ml), 32.41 ( $3 \times 10^4$  CFUs/ml), and 35.44 ( $3 \times 10^3$  CFUs/ml). Fecal-positive samples inoculated with concentrations from  $10^2$  to  $10^5$  CFUs/ml were also detected by RT PCR with mean  $C_t$  values of 26.67 ( $5 \times 10^5$  CFUs/ml), 29.60 ( $5 \times 10^4$  CFUs/ml), 33.67 ( $5 \times 10^3$  CFUs/ml), and 35.45 ( $5 \times 10^2$  CFUs/ml) with one isolate negative at this concentration. After enrichment, both fecal-positive and fecal-negative samples originally inoculated with concentrations from  $10^0$  to  $10^5$  CFUs/ml were all detected by both RT PCR and culture. The mean  $C_t$  value for all fecal-negative spiked samples postenrichment was 15.45 and ranged from 15.1 to 15.9, regardless of initial concentration. Fecal-positive spiked samples had a  $C_t$  value

range of 17.33 ( $5 \times 10^5$  CFUs/ml) to 24.67 ( $5 \times 10^0$  CFUs/ml). Negative controls did not generate  $C_t$  values.

**Field study.** Of the 208 samples collected from commercial layer houses, SE was detected by culture in 7 (3.4%) samples. A  $C_t$  value was obtained in 20 (9.6%) samples with RT PCR testing (Fig. 1). The 7 culture-positive samples had a mean  $C_t$  value of 20.0 ( $C_t$  range 15.7–24.3), while those 13 positive by RT PCR but culture-negative had a mean  $C_t$  value of 37.2 ( $C_t$  range 32.9–39.4). The difference between means was 17.2 cycles (95% CI 14.9–19.4,  $P < 0.0001$ ).

**Optimum cutoff values from TG ROC plots.** On the TG ROC plots, the curves for sensitivity and specificity intersected at 100% for each individual and pooled sample set (Fig. 2). Because the point of intersection was at 100% for sensitivity and specificity curves, all cutoff values determined from the TG ROC plot were the optimum cutoff for their respective group of samples. In the individual samples, sensitivity and specificity reached 100% at a  $C_t$  value of 24.3. Likewise, the pools of two, three, and four all reached 100% sensitivity and specificity at  $C_t$  values of 27.3, 24.3, and 27.9, respectively.

**Agreement among cutoffs.** Agreement between individual and pooled RT PCR results versus culture results varied at different  $C_t$  cutoff levels (Table 3). When no  $C_t$  cutoff ( $\leq 40$ ) was applied, the percentage agreement between culture and RT PCR among individual samples was 93.8% ( $\kappa = 0.49$ ). For pooled samples, the percentage of agreement with culture was 97.1% ( $\kappa = 0.81$ ), 95.7% ( $\kappa = 0.80$ ), and 98.1% ( $\kappa = 0.91$ ) for the pools of two, three, and four, respectively. Various cutoff values were examined to assess the level of agreement between culture and RT PCR. Agreement with RT PCR for individual samples was 99% ( $\kappa = 0.87$ ) at a  $C_t$  cutoff of 36, 100% ( $\kappa = 1.00$ ) at a  $C_t$  cutoff of 30, and 100% ( $\kappa = 1.00$ ) at a  $C_t$  cutoff of 28. For pooled samples with at least one culture-positive sample at  $C_t < 36$ , agreement was 98.1% ( $\kappa = 0.87$ ), 97.1% ( $\kappa = 0.86$ ), and 98.1% ( $\kappa = 0.91$ ) for pools of two, three, and four, respectively. Likewise at  $C_t < 30$ , agreement was 99.0% ( $\kappa = 0.93$ ), 100% ( $\kappa = 1.00$ ), and 100% ( $\kappa = 1.00$ ). At a cutoff of 28, all sets of individual and pooled samples had 100% ( $\kappa = 1.00$ ) agreement between culture and RT PCR.

## DISCUSSION

The results of the enrichment study suggested that the detection limit of the RT PCR assay was markedly improved using the tetrathionate enrichment. Without enrichment a bacterial load of approximately  $10^3$  CFUs/ml was needed to be reliably detected by RT PCR. However, after enrichment, samples inoculated with as little as 1 CFU/ml were detectable. All fecal-negative samples initially inoculated with SE at concentrations between  $10^0$  and  $10^5$  CFUs/ml and subsequently enriched in tetrathionate had a similar  $C_t$  value existing within the range of 15.1 and 15.9. This indicates that the enrichment promoted SE growth to a point that likely saturated the PCR reaction in all samples. Fecal-positive inoculated samples had higher  $C_t$  values after enrichment at each concentration than the fecal-negative samples with a range between 17.3 and 24.7 but still had  $C_t$  values markedly lower than samples tested prior to enrichment. For comparison, nonenriched samples containing approximately  $10^8$  CFUs/ml of SE gave  $C_t$  values that averaged 17.3 while those containing approximately  $10^6$  CFUs/ml of SE gave  $C_t$  values that averaged 25.5. Considering the fact that fecal-negative enriched samples gave  $C_t$  values between 15.1 and 15.9 and fecal-positive samples gave  $C_t$  values between 17.3 and 24.7, it appears that enrichment favored the growth of SE within each sample to a minimum of  $10^6$  CFUs/ml and potentially higher than  $10^8$  CFUs/ml.

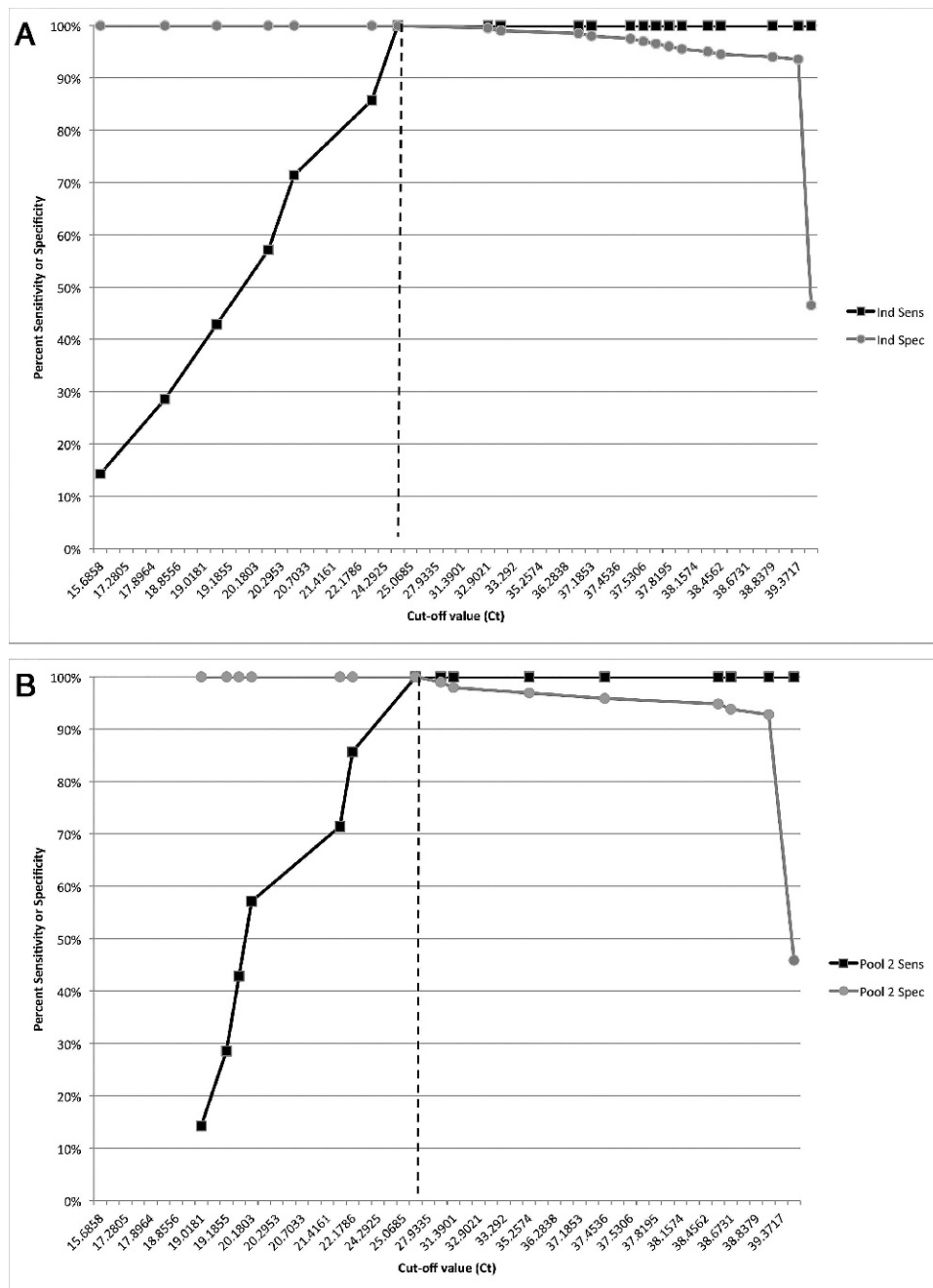


Fig. 2. Two-graph ROC plot of individual, pool of two, pool of three, and pool of four samples. Optimal cutoff values at 100% sensitivity and specificity were 24.3 for individual samples (A), 27.3 for pool of 2 (B), 24.3 for pool of 3 (C), and 27.9 for pool of four (D).

ml regardless of the initial level of inoculum. At this level, RT PCR and culture are able to easily confirm the sample as positive. Nam *et al.* documented the same observation, noting that the detection limit of their RT PCR assay improved from  $10^3$  to  $10^4$  CFUs/ml to 10 CFUs/ml of inoculum after enrichment (14). The addition of chicken feces to the sample did not appear to affect the RT PCR assay on samples tested before enrichment but may have been a cause for the increased  $C_t$  values found on samples tested after enrichment. It is known that feces contain bile salts and complex polysaccharides, which could potentially inhibit SE growth and/or the RT PCR causing the increased  $C_t$  values observed after enrichment (12).

Among the positive samples in the field study, there appeared to be two distinct populations. One population had low  $C_t$  values ( $C_t$

range 15.7–24.3) and consisted of samples that tested positive by culture and PCR. The second population had distinctly higher  $C_t$  values ( $C_t$  range 32.9–39.4) and was composed of samples testing positive by PCR but negative by culture. The population of culture-positive, RT PCR-positive samples ( $C_t$  range 15.7–24.3) presented  $C_t$  values in line with level of detection results showing inoculated samples had similar  $C_t$  values ( $C_t$  range 15.1–15.9). There are a number of possible reasons for the detection of SE DNA in PCR-positive, culture-negative samples. One possible reason is that there was nonspecific amplification of background nucleic acids or degradation of the probe-based fluorophore (6,7), that is, a false positive. Another possibility is that the sample contained dead or nonviable cells that were unable to grow in culture but were



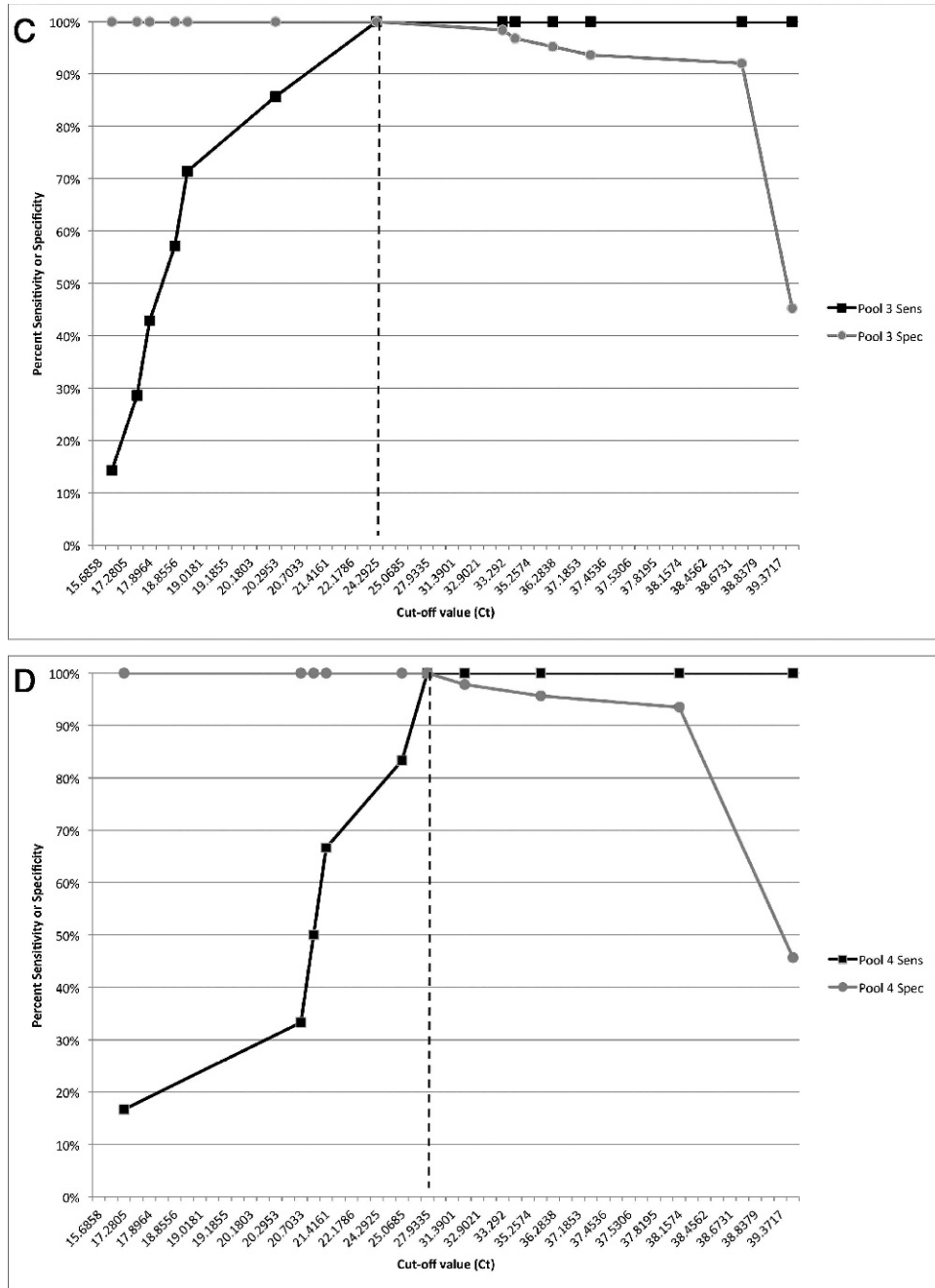


Fig. 2. Continued.

detectable by PCR (18), that is, a false positive when compared to “gold standard” by the NPIP test.

To maximize the level of agreement between culture and RT PCR among individual and pooled samples, the optimum  $C_t$  cutoff was determined using a TG ROC plot. Cycle threshold cutoff values selected from the TG ROC plot improved the level of agreement between bacterial culture for *Salmonella* and SE-specific RT PCR to 100% in all individual and pooled sample sets. These cutoff values improved the agreement by eliminating the population of samples that tested PCR-positive but culture-negative. All samples in the population that tested positive by both culture and RT PCR were unaffected by the cutoff. While the true SE status of PCR-positive, culture-negative samples is unknown, their weak  $C_t$  values and

failure to detect SE by culture after enrichment suggest that they are unlikely to contain viable SE.

Table 3. Percentage of agreement between samples, individual or pooled, that were positive by culture testing and RT PCR at various levels of cutoff values.

Agreement	No cutoff	$C_t \leq 36$	$C_t \leq 30$	$C_t \leq 28$
Individual	93.8%	99.0%	100.0%	100.0%
Pool of 2	97.1%	98.1%	99.0%	100.0%
Pool of 3	95.7%	97.1%	100.0%	100.0%
Pool of 4	98.1%	98.1%	100.0%	100.0%

Optimum cutoff values determined from the TG ROC plots differed among each individual and pooled sample set and were calculated separately; however, this method would be impractical in regular diagnostic use. Therefore, establishing a standard cutoff value is preferable. There was a high agreement (>93%) between culture and RT PCR for all individual and pooled sample sets. The percentage agreement among individual and pooled samples increased as the  $C_t$  cutoff decreased from 36, to 30, and finally to 28. These results are in accord with a similar study conducted using a PCR assay based on the *Salmonella invA* gene (18). At a cutoff of 28, the percentage agreement between culture and RT PCR results was 100% for all sample sets. The cutoff level of 30 had only one discordant sample. This discordant sample had a  $C_t$  of 28.13, which is a midrange value. The true SE status of this sample is hard to determine since it has the lowest  $C_t$  value among the PCR-positive, culture-negative samples but approaches the highest  $C_t$  value of the PCR-positive, culture-positive samples. The ambiguity of this sample prevents the total rejection of 30 cycles as a plausible cutoff. In a practical setting, a cutoff value of  $\leq 36$  would be acceptable using the protocol employed in this study. It is likely that some samples would be considered positive by RT PCR and negative by culture at this level. However, it is expected that culture confirmation would still be used to determine the final status of RT PCR-positive samples.

In conclusion, the agreement between individual and pooled sample sets was similar across cutoff values. Given these results, it can be concluded from this study that RT PCR testing of pooled samples is equivalent to RT PCR testing of individual samples and individual testing by culture. Sample pooling was effective in detecting SE in each pool of two, three, and four. Enrichment was an important step in improving the detection limit of the RT PCR assay and thus in increasing its sensitivity. With enrichment, any SE present was amplified to an easily detectable low  $C_t$  value regardless of initial concentration. High  $C_t$  values therefore are questionable in regard to their correlation to positive culture results. Cutoff values can be selected that improve the correlation to culture status of SE-specific RT PCR results by eliminating results above the  $C_t$  cutoff. Following enrichment in tetrathionate, RT PCR testing of pooled samples created by combining two, three, and four individual samples was equivalent to culture testing of individual samples and is potentially useful as a detection test of poultry environments.

## REFERENCES

1. 9 CFR 147.12. Procedures for collection, isolation, and identification of *Salmonella* from environmental samples. U. S. National Archives and Records Administration, pp. 946–952. 2011.
2. Andrews, W. H. Evolution of methods for the detection of *Salmonella* in foods. *J. AOAC Int.* 79:4–12. 1996.
3. Angulo, F. J., and D. L. Swerdlow. Epidemiology of human *Salmonella enterica* serovar Enteritidis infections in the United States. Iowa State University Press, Ames, IA. 1999.
4. Applied Biosystems. Taqman *Salmonella* Enteritidis detection kit protocol for drag swabs: protocol for real-time PCR detection of SE in environmental samples. Life Technologies, Carlsbad, CA. p. 18. 2010.
5. Boraychuk, V. M., G. E. Gensler, M. E. McFall, R. K. King, and D. G. Renter. A real-time PCR assay for the detection of *Salmonella* in a wide variety of food and food-animal matrices. *J. Food Prot.* 70:1080–1087. 2007.
6. Burns, M., and H. Valdivia. Modelling the limit of detection in real-time quantitative PCR. *Eur. Food Res. Technol.* 226:1513–1524. 2008.
7. Caraguel, C. G. B., H. Stryhn, N. Gagne, I. R. Dohoo, and K. L. Hammell. Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: analytical and epidemiologic approaches. *J. Vet. Diagn. Invest.* 23:2–15. 2011.
8. Catarama, T. M. G., K. A. O'Hanlon, D. A. McDowell, I. S. Blair, and G. Duffy. Comparison of a real-time polymerase chain reaction assay with a culture method for the detection of *Salmonella* in retail meat samples. *J. Food Saf.* 26:1–15. 2006.
9. Dohoo, I., W. Martin, and H. Stryhn. Veterinary Epidemiologic Research. AVC Inc., University of Prince Edward Island, Charlottetown, Canada. 85–120. 2003.
10. FDA Federal Register. Prevention of *Salmonella* Enteritidis in shell eggs during production, storage, and transportation; final rule. Department of Health and Human Services, Washington, DC. pp. 33030–33101. 2009.
11. Humphrey, T. J., A. Baskerville, S. Mawer, B. Rowe, and S. Hopper. *Salmonella*-enteritidis phage type-4 from the contents of intact eggs: a study involving naturally infected hens. *Epidemiol. Infect.* 103:415–423. 1989.
12. Inglis, G. D., and L. D. Kalischuk. Use of PCR for direct detection of *Campylobacter* species in bovine feces. *Appl. Environ. Microbiol.* 69:3435–3447. 2003.
13. Malorny, B., C. Bunge, and R. Helmuth. A real-time PCR for the detection of *Salmonella* enteritidis in poultry meat and consumption eggs. *J. Microbiol. Meth.* 70:245–251. 2007.
14. Nam, H. M., V. Srinivasan, B. E. Gillespie, S. E. Murinda, and S. P. Oliver. Application of SYBR green real-time PCR assay for specific detection of *Salmonella* spp. in dairy farm environmental samples. *Int. J. Food Microbiol.* 102:161–171. 2005.
15. Patrick, M. E., P. M. Adcock, T. M. Gomez, S. F. Altekruse, B. H. Holland, R. V. Tauxe, and D. L. Swerdlow. *Salmonella* enteritidis infections, United States, 1985–1999. *Emerg. Inf. Dis.* 10:1–7. 2004.
16. Preliminary foodNet data on the incidence of infection with pathogens transmitted commonly through food: 10 states, 2009. *Morb. Mortal. Wkly Rep.* 59:418–422. 2010.
17. Seo, K. H., I. E. Valentin-Bon, R. E. Brackett, and P. S. Holt. Rapid, specific detection of *Salmonella* enteritidis in pooled eggs by real-time PCR. *J. Food Prot.* 67:864–869. 2004.
18. Singer, R. S., C. L. Cooke, C. W. Maddox, R. E. Isaacson, and R. L. Wallace. Use of pooled samples for the detection of *Salmonella* in feces by polymerase chain reaction. *J. Vet. Diagn. Invest.* 18:319–325. 2006.
19. Tomas, D., A. Rodrigo, M. Hernandez, and M. A. Ferrus. Validation of real-time PCR and enzyme-linked fluorescent assay-based methods for detection of *Salmonella* spp. in chicken feces samples. *Food Anal. Meth.* 2:180–189. 2009.
20. Waltman, W. D. Methods for isolating salmonellae from poultry and the poultry environment. Iowa State University Press, Ames, IA. 1999.

## ACKNOWLEDGMENTS

The authors want to thank Joann Kinyon of the Iowa State University Diagnostic Lab and Applied Biosystems for their assistance with this study.