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Application of Nested Polymerase Chain Reaction to Detection of *Salmonella* in Poultry Environment

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ABSTRACT

Isolation of *Salmonella* from environmental and processing-plant poultry samples requires the sampling of large numbers of areas within the poultry house or plant. Subsequently, the required number of samples necessitates a large volume of work for a microbiology laboratory, especially when the protocol requires the inclusion of a delayed secondary enrichment for the isolation of *Salmonella.* This study examined the use of the polymerase chain reaction (PCR) to identify those secondary enrichments containing *Salmonella.* The unique *Salmonella* virulence gene *invA* was chosen as the target for the development of a nested PCR because of its uniform distribution among *Salmonella* serotypes. The use of nested PCR primers increased the sensitivity of detection 100-fold, resulting in the detection of as few as four cells. There was a strong, statistically significant positive correlation between PCR and culture results as determined by chi-square ($P < 0.001$) and kappa ($k = 0.915$; excellent agreement) tests. Using PCR to screen primary enrichments for presumptive *Salmonella* contamination, we improved our efficiency at isolating *Salmonella* upon secondary enrichment by 20%, and no false negatives were observed. This method will not only validate the use of secondary enrichment procedures but also reduce costs and manpower required for the surveillance of *Salmonella.*

Salmonella is one of the leading causes of outbreak-associated gastroenteritis in the United States *(36).* Food poisoning associated with this organism is primarily due to the consumption of meat, egg, and milk products tainted with *Salmonella,* although many other vehicles have recently been recognized *(36).* The consumption of poultry has been implicated in 40% of food-poisoning outbreaks attributed to *Salmonella* in the United States *(36).* Because of public concern, the Food Safety and Inspection Service mandated that meat-processing plants implement hazard analysis critical control point programs and meet U.S. Department of Agriculture–approved limits for *Salmonella* contamination of poultry and other meat products *(3).* Levels of *Salmonella* contamination of poultry products were reported to be 10.8% in 1998, 11.4% in 1999, and 9.1% in 2000 (http://www.usda.gov). Some consumer groups have called for additional measures to further reduce the levels of *Salmonella* entering processing plants through live animals. "The earlier in the food chain that pathogens are controlled, the less chance that consumers will be exposed with the potential for illness or death," said Caroline Smith DeWaal, Director, Center for Science in the Public Interest *(43).* Some methods to further reduce *Salmonella* contamination on the final product may require on-farm intervention strategies.

The prevention of the introduction and reintroduction of *Salmonella* into poultry flocks is a key strategy in the control of *Salmonella* contamination *(30).* The biosecurity strategies employed to prevent the introduction of *Salmonella* can be similar to those employed to prevent the introduction of diseases that impact the health of livestock and poultry *(23, 41).* Because *Salmonella* is endemic on many food animal farms, it is necessary to enhance the ability of the animals to resist *Salmonella* infection or colonization through the use of probiotics, vaccines, or antibiotics *(22, 26, 31, 34, 39).* Evaluation of the success of an on-farm intervention strategy will require rigorous monitoring of the environment for *Salmonella.*

Current monitoring schemes involve selective bacterial culture enrichment and subsequent screening for H_2S-producing non-lactose-fermenters. Delayed secondary enrichment culture has been used to improve the detection of *Salmonella* in samples *(49)* and is required for poultry companies participating in the National Poultry Improvement Plan *(4).* This culture technique requires additional screens involving differential-selective media like XLT4 and brilliant green agar (BGN). *Salmonella* isolation can be expensive and labor intensive when the large number of samples necessitated by the National Poultry Improvement Plan for *Salmonella* reduction in poultry flocks *(3)* must be processed.

Polymerase chain reaction (PCR), a molecular technique, is widely used in the identification of foodborne pathogens, and it has become an invaluable diagnostic tool in clinical microbiology *(27, 28).* PCR has been adapted for the rapid detection of *Salmonella* in foods *(10, 13, 42),*

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in the environment *(17, 18, 44),* and in clinical specimens *(12, 14, 18, 40, 45).* Several PCR tests have been developed on the basis of DNA sequence(s) of the *Salmonella* virulence genes *(1, 10, 12–15, 17, 18, 40, 42, 44, 45).* However, the uneven distribution of several of these genetic markers within this bacterial population makes tests based on them less useful in screening for *Salmonella (6, 46).* Only PCR tests involving *Salmonella* invasion gene sequence(s) have proved successful as universal tests for *Salmonella* because of the uniform distribution of the *inv* genes among various *Salmonella enterica* serotypes *(20, 46).* These PCR tests are based on the *Salmonella invA* DNA sequence *(1, 10, 12–14, 17, 18, 40, 45, 46),* a type III secretion pathway component that is strongly conserved but unique to *Salmonella (9, 20).*

In order to simplify *Salmonella* isolation with delayed secondary enrichment, a nested PCR was developed to identify positive enrichment broths prior to plating on selective agar. *Salmonella* virulence gene *invA* was chosen as the target for nested PCR because it is well conserved and specific for *Salmonella (20, 46).* The correlation between PCR and culture detection was determined.

MATERIALS AND METHODS

Enrichment. The environments of four commercial broiler houses were sampled for *Salmonella* with drag swabs, gauze pads soaked with double-strength skim milk and then dragged across the birds' bedding material *(11).* Swabs (n = 188) were then placed in 100 ml of tetrathionate brilliant green broth (TTB; Difco Laboratories, Detroit, Mich.) and incubated at 41.5°C for 18 h *(7).* Chicken carcasses (n = 80) collected at the processing plant prior to and immediately after placement in the chlorine-containing ice water chilling tank were rinsed with 250 ml of buffered peptone water *(49).* These carcass rinses were used to inoculate TTB, which was subsequently incubated at 41.5°C for 18 h.

***Salmonella* isolation and identification.** A loop full of the TTB enrichment broth was streaked onto a XLT4-BGN biplate (Difco) and incubated at 37°C overnight *(21).* Four to five isolated H_2S-positive colonies were used to inoculate triple sugar iron slants (Difco), which were incubated overnight at 37°C. Suspect *Salmonella* colonies were confirmed with poly O *Salmonella*-specific antiserum (Difco).

DNA extraction. One milliliter of TTB enrichment broth was centrifuged at 100 × g for 5 min to remove the calcium carbonate particles. The supernatant, containing bacteria, was transferred to a second tube and centrifuged at 4,500 × g for 10 min. The resulting bacterial pellet was resuspended in 1 ml of water, washed once with water, and heated in a dry block at 100°C for 10 min. The lysate was then centrifuged for 10 min at 4,500 × g to pellet debris. The supernatant containing DNA was used as a template in the PCR.

Nested PCR. Two amplifications were performed to improve the detection of *Salmonella* by PCR. Both PCR reactions were carried out with the Rapidcycler hot-air thermocycler (Idaho Technology, Salt Lake City, Utah) *(50).* A 10-μl PCR reaction was prepared by loading 9 μl of PCR reaction mix and 1 μl of DNA template into a 0.2-ml flat-cap PCR tube. The PCR reaction mix consisted of 3 mM $MgCl_2$, 50 mM Tris (pH 7.4), 0.25 mg of bovine serum albumin per ml, 1.25 μM oligonucleotide primer, 0.2 mM deoxynucleoside triphosphate (Boehringer Mannheim, Indianapolis, Ind.), and 0.5 U of *Taq* polymerase (Boehringer Mannheim) for both amplifications. The two primer sets used in this study were designed from the *Salmonella invA* virulence gene by using the DNA software analysis program Generunner 3.1 (Hastings Software Inc., Hastings-on-Hudson, N.Y.), and oligonucleotides were synthesized at the Molecular Genetics Instrumentation Facility at the University of Georgia with the ABI Model 394 synthesizer. The sequences for the four nested PCR primers are shown in Table 1. The first amplification involved 30 cycles of 93°C for 1 s, 42°C for 1 s, and 72°C for 15 s with a slope of 2 using primers *invA*F and *invA*R. The second (nested) PCR involved 30 cycles of 94°C for 10 s, 55°C for 10 s, and 72°C for 35 s with a slope of 2 using primers *invA*nestF and *invA*nestR.

TABLE 1. *PCR primers*

Primer	Sequence	Expected size (bp)
*invA*1	F: CTGTTGAACAACCCATTTGT	437
*invA*2	R: CGGATCTCATTAATCAA-CAAT	
*invA*nest1	F: AACCAGCAAAGGCGAGCAG	199
*invA*nest2	R: GCGCACGCCATAATCAA-TAAA	

Comparison of detection limits of nested PCR and single PCR. Tenfold serial dilutions were made from extracted DNA of a *Salmonella* Typhimurium broth culture grown overnight in Luria broth. Two PCR amplifications were then performed for these samples following the procedures described above, where 1 μl from the first PCR reaction served as the template for the second (nested) PCR reaction. The PCR products were detected by electrophoresis in 1.5% agarose gel with 0.2 μg of ethidium bromide per ml at 100 V for 30 min. The 100-bp ladder (Gibco/BRL, Gaithersburg, Md.) was used as a molecular weight marker to determine the molecular weight of PCR products.

In experiment 1, PCR was carried out only for primary enrichments for drag swabs (n = 141) or carcass rinses (n = 80). Delayed secondary enrichment was carried out for samples that were PCR positive but culture negative. In experiment 2, primary and delayed secondary enrichment was carried out for all drag swabs (n = 47), regardless of culture results. PCR was carried out for all primary and secondary enrichments.

Delayed secondary enrichment. Following initial plating, the original TTB culture enrichment broth was left on the bench at room temperature for 5 days *(48, 49).* After this 5-day incubation, 1 ml from each of the culture samples that were PCR positive but culture negative was transferred to 10 ml of fresh TTB, incubated at 37°C overnight, and subsequently plated *(48).*

Sensitivity of TTB in enrichment of *Salmonella*. To simulate the normal background microflora in a poultry environmental sample, 225 g of litter was collected from two chicken pens in an experimental poultry house at the University of Georgia and pooled together. Five grams of the litter sample was aliquoted into each of the 45 centrifuge tubes (50-ml capacity). Thirty milliliters of phosphate-buffered saline was added to each sample. After samples were shaken with a wrist-action shaker (Burrell, Pittsburgh, Pa.) at the maximum setting for 5 min, the samples were centrifuged at 100 × g for 15 min to pellet debris. The supernatant, containing suspended bacteria, was transferred to new 50-ml tubes and centrifuged at 4,500 × g to pellet bacteria. The pellets were pooled and resuspended in 10 ml of superbroth with 15%

glycerol and stored at −70°C. One hundred microliters of this litter miroflora was spread onto MacConkey agar with rifampicin (100 μg/ml) and incubated overnight at 37°C to determine if there were rifampicin-resistant bacteria already present in the litter. The presence of *Salmonella* with litter microflora was assessed by nested PCR. A rifampicin-resistant *Salmonella* Typhimurium was used to inoculate poultry house litter. An isolated colony was inoculated into superbroth *(38)* and incubated at 37°C overnight. Tenfold serial dilutions were made from the *Salmonella* culture. To simulate normal poultry house culture procedures, 100 ml of TTB and 0.5 ml of litter bacterial stock were added to each of 18 sterile plastic bags and divided into two groups (with and without *Salmonella*). One milliliter from each *Salmonella* dilution was used to inoculate the contents of nine of the bags with litter microflora. The bags were then incubated at 41.5°C overnight. The overnight cultures were streaked onto XLT4 and BGN plates. One hundred microliters of undiluted broth from each of the culture enrichments was spread onto MacConkey agar with rifampicin (100 μg/ml). Tenfold serial dilutions were made from each TTB enrichment broth onto MacConkey agar with rifampicin (100 μg/ml). For final confirmation that the reisolated *Salmonella* was the inoculated *Salmonella,* an agglutination test with *Salmonella* O group B factor–specific antiserum was carried out for suspect *Salmonella* colonies on XLT4 and MacConkey agar-plus-rifampicin plates.

Statistics. Because culture, actual isolation, and identification of the organism is the established technique for identifying *Salmonella* present in environmental samples, it was assumed to be the "gold standard" test. The positive and negative predictive values, sensitivity, specificity, and test agreement value (k) were calculated with the statistical program SAS 8.1 (SAS Institute Inc., Cary, N.C.). Chi-square values at $P = 0.001$ were also calculated for each of the contingency tables.

RESULTS

Sensitivity of *invA* nested PCR for detecting *Salmonella*. The sensitivities of single PCR and nested PCR were evaluated by using serial dilutions of *Salmonella* Typhimurium template starting with 4.3×10^7 CFU/ml (Fig. 1). PCR products of 437 and 199 bp from the first amplification and the second (nested) amplification, respectively, could be observed. The first PCR detected as few as 430 cells. The nested PCR was able to detect 4 cells, representing a 100-fold increase in the detection limit. In other words, the nested PCR can detect *Salmonella* at a density of 4,000 cells per ml. Coupled with primary enrichment, would PCR be sensitive enough to detect *Salmonella*?

Sensitivity of TTB culture enrichment. The concentration of undiluted overnight culture of rifampicin-resistant *Salmonella* Typhimurium was 4×10^7 CFU/ml. There were no colonies observed on MacConkey agar-plus-rifampicin, BGN, and XLT4 plates for the negative control, TTB with poultry litter microflora only. *Salmonella* was detected on MacConkey agar-with-rifampicin, BGN, and XLT4 plates at every dilution (10^{-1} to 10^{-7}) of *Salmonella* Typhimurium spiked in the TTB with poultry litter microflora. The presence of litter microflora did not interfere with the detection of rifampicin-resistant *Salmonella* Typhimurium by culture. The detection limit for *Salmonella* in litter with TTB enrichment was 1 CFU/ml. Following overnight enrichment, *Salmonella* Typhimurium grew to a final density of 10^5 to 10^7 CFU/ml in each tube of TTB, regardless of the initial inocula, as determined from colony counts on MacConkey agar plus rifampicin. In spite of competing microflora, initial enrichment yielded sufficient cell density for the detection of *Salmonella* by PCR, starting with one *Salmonella* cell per 100 ml of TTB.

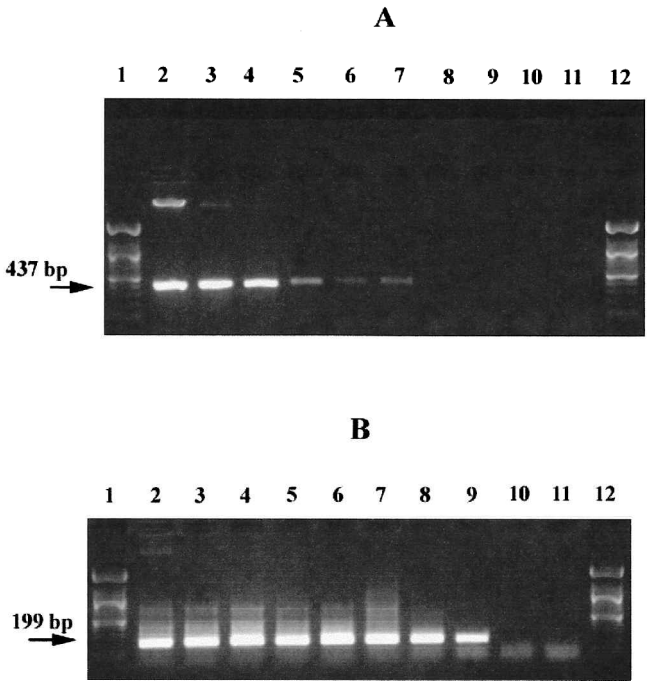

FIGURE 1. *Detection limit of nested PCR for* Salmonella. *(A) First primer set. (B) Nested primer set. Lanes 1 and 12, 100-bp ladder as molecular weight marker; lane 2, 4.3×10^7 cells; lane 3, 4.3×10^6 cells; lane 4, 4.3×10^5 cells; lane 5, 4.3×10^4 cells; lane 6, 4.3×10^3 cells; lane 7, 4.3×10^2 cells; lane 8, 4.3×10^1 cells; lane 9, 4.3×10^0 cells; lane 10, 4.3×10^{-1} cells; lane 11, no DNA template control.*

PCR as a screen for identifying *Salmonella*-positive samples following enrichment in TTB. One hundred forty-one drag swabs and 80 carcass rinses were collected from four poultry houses and from flocks processed at these sites. Of those samples from the primary enrichment that were PCR positive ($n = 73$), 42 were also culture positive (Table 2). Delayed secondary enrichments were carried out for negative primary enrichments, which were PCR positive. From this second enrichment, 10 samples were culture positive for *Salmonella.*

To further validate nested PCR as a screen for determining which samples need to be directed to secondary enrichment, a second experiment was designed to evaluate the correlation between PCR and culture results. Forty-seven drag swabs from the same four poultry houses were subjected to primary enrichment and subsequent delayed secondary enrichment. PCR was performed for the primary enrichments only. Of the 28 samples that tested positive for *Salmonella* by PCR, 22 were also culture positive. From delayed secondary enrichment, we were able to isolate *Salmonella* from four additional samples that initially tested positive for *Salmonella* by PCR after primary enrichment.

TABLE 2. *Comparison of nested PCR with the culture method for detecting* Salmonella

PCR (primary enrichment)	Primary enrichment (%)		Delayed secondary enrichment (%)	
	Culture positive	Culture negative	Culture positive	Culture negative
Experiment 1[a]				
PCR positive	42/221 (19)	31/221 (14)	10/31 (32)	21/31 (68)
PCR negative	0/221 (0)	148/221 (67)	ND[b]	ND
Experiment 2[c]				
PCR positive	22/47 (47)	5/47 (11)	26/47 (55)	2/47 (4)
PCR negative	0/47 (0)	20/47 (42)	0/47 (0)	20/47 (42)

[a] Delayed secondary enrichment was performed for samples that were PCR positive but culture negative from primary enrichment.
[b] ND, not determined.
[c] Delayed secondary enrichment was performed for all samples regardless of the PCR result.

No samples that tested negative for *Salmonella* by PCR ever tested positive by culture. There were two samples that tested positive for *Salmonella* by PCR, but we were unable to isolate the organism after secondary enrichment. When nested PCR was compared with culture (as the gold standard), we found no false negatives, 4.3 and 9.5% false positives for the two experiments, and excellent statistical correlation between PCR and culture results, as is evident from the positive and negative predictive values and the chi-square test ($P < 0.001$) (Table 3).

DISCUSSION

An effective program to eradicate foodborne pathogens like *Salmonella* will eventually require not only the adoption of a hazard analysis critical control point program at the processing plant, but also the implementation of an on-farm intervention strategy. At the processing plant, rapid detection methods for biological hazards are needed to determine the relative safety of products before they reach the consumer. Although several different rapid tests have been developed for detecting harmful pathogens on foods *(19, 37),* culture is still the universally accepted method of identifying contamination of foods with significant bacterial pathogens like *Salmonella.* On-farm intervention strategies often rely on the same culture methods used in processing plants for detecting pathogens like *Salmonella* but do not have the same time constraints for the processing of samples. However, the implementation of a *Salmonella* monitoring program, as recommended by National Poultry Improvement Plan, will require the processing of larger sample sizes over longer periods.

TABLE 3. *Comparison of sensitivity and specificity values for nested PCR and the culture method*

Parameter	Value	Evaluation
Positive predictive value	0.95	Excellent
Negative predictive value	0.95	Excellent
Relative sensitivity[a]	0.96	Excellent
Relative specificity[b]	0.95	Excellent
Kappa (test agreement)	0.915	Excellent
Chi-square	$P < 0.001$	Significant

[a] Measure of false negatives.
[b] Measure of false positives.

The detection of *Salmonella* in either foods or environmental samples often requires an enrichment step followed by the isolation of the organism on a differential-selective medium. Several enrichment media have been developed for isolating *Salmonella.* These media differ with regard to their abilities to isolate this organism from food *(16),* animal waste *(5),* and the environment *(8, 32, 33, 35, 47).* The sensitivity of the culture method can be improved by 42% by using secondary enrichment, but this improvement comes at the cost of an enrichment time increase of 5 days *(48, 49).* In this study, we used PCR to determine which primary enrichments contained *Salmonella,* and we used those samples for culture isolation of the organism.

In order to develop a PCR for use as a screen of preenrichments for *Salmonella,* it is important to have a sensitive test. With nested PCR, we were able to detect as few as four cells, demonstrating a sensitivity similar to those of PCRs reported by other labs *(40, 45).* Because PCR is carried out with microliter volumes, this sensitivity can be extrapolated to the detection of *Salmonella* at 10^3 CFU/ml. However, by using enrichment and culture, we could detect as few as 1 CFU of *Salmonella* per ml, representing a sensitivity similar to the culture sensitivities reported by others using similar preenrichment protocols *(24, 25).* The greater sensitivity of the culture method is due to the fact that the sample volumes processed by culture methods are larger than those processed by PCR, for which reaction volumes seldom exceed 100 μl. Because of this limitation, an initial preenrichment culture has been incorporated into many PCR-based schemes for detecting *Salmonella* in order to increase sensitivity *(12–14, 18, 40, 42, 45).* With our protocol, tetrathionate broth inoculated with as few as 1 CFU/ml of *Salmonella* yielded 10^5 CFU/ml following overnight incubation at 41°C. This concentration of cells in the preenrichment is well within the detection limit of our nested PCR.

Most studies comparing detection techniques have not statistically compared PCR with the gold standard culture method with regard to the detection of *Salmonella.* In these studies, specificity and sensitivity are usually defined in molecular biological, not statistical, terms *(10, 13, 14, 17,*

40, 45). We were interested in statistically determining whether PCR was indeed a valid predictor of cultivable *Salmonella* in the poultry environment. Because the desired result was to recover *Salmonella* from all samples, test sensitivity was favored over specificity. For our purposes, test sensitivity was defined as the probability that a test would show a truly positive sample to be positive. In general, sensitivity and specificity are inversely related. Because no data are available on the sensitivity and specificity of the primary and secondary culture tests used as the gold standard, the calculated sensitivity and specificity of the PCR are reported as relative sensitivity and specificity. When the nested PCR was compared with the culture method with regard to the ability to detect *Salmonella,* excellent test sensitivity (96%) and excellent test specificity (95%) were seen. No false negatives were observed, which is an important finding because it enables us to confidently focus culture or secondary enrichments primarily on only those samples that are PCR positive. Our findings contrast with those of Carli et al. *(12),* who reported a large number of false negatives with PCR they developed. In this study, of the 18 PCR-negative samples, 14 were culture positive after delayed secondary enrichment. The discrepancy between our results and those of Carli et al. is most likely due to the design of the *invA*-PCR primers. We focused our primer design on highly conserved sequences within *invA (9).* Soumet et al. *(44)* also reported false negatives with their PCR, but the discrepancy between PCR and culture results appeared to be due more to the limited distribution of the PCR target genes, *sefA* and *fliC*$_i$, among *Salmonella.* For this study, the test agreement value k was calculated to be 0.8, indicating good test agreement between the culture method and PCR. Another study also demonstrated good statistical agreement between the culture method and PCR ($k = 0.76$) provided that a preenrichment step was included with PCR, and in this study, the test agreement also varied with the medium used in the preenrichment step *(18).* The Achilles' heel of this and other tests consists in samples identified by PCR as positive for *Salmonella* being culture negative following primary or secondary enrichment *(2, 12, 14, 40, 44).* False positives reported for these PCR tests range from 5% *(14)* to 95% *(2).* In two separate experiments, we found the percentages of samples that were PCR positive and culture negative to range from 7 to 29%. In this study, the differences in false positives observed for experiments 1 and 2 appear to be attributable mainly to sample size. False-positive results are less likely because of the production of nonspecific PCR amplicons, since nested PCR has greater specificity because of the inclusion of a second set of primers internal to the first set, which can only anneal if the first primer set has amplified the correct target gene sequence *(15).* Other investigators have used similar tactics in demonstrating the specificity of their PCR for *Salmonella (2, 45).* In order to verify the PCR, we sequenced the 199-bp nested PCR amplicon from a PCR-positive, culture-negative sample, and the DNA sequence had 99.99% identity with *Salmonella* Typhimurium *invA,* suggesting that there are uncultivable *Salmonella* in the sample. Others have explained the discrepancies between their PCR and culture results as being due to the inability of PCR to distinguish live from dead cells, but differences in enrichment, differential-selective media, and culture conditions might also explain PCR-positive, culture-negative samples *(44)* as well as a failure to recognize atypical *Salmonella* colony types on differential-selective agars *(18, 44).*

This study provides further support for the utility of PCR as a diagnostic tool in initial screens of poultry samples for *Salmonella* contamination. By using nested PCR as our screen, we improved our efficiency at isolating *Salmonella* upon secondary enrichment and presumptively identifying samples contaminated with this organism by 20%. Through initial screening, culture isolations can be limited to those samples that are PCR positive, saving the time and materials required for the processing of large sample sizes. Currently, we are developing a PCR enzyme-linked immunosorbent assay *(29)* for detecting *Salmonella* following primary enrichment in order to further streamline the screening process.

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