INTEGRATION OF HYDROXYAPATITE CONCENTRATION OF BACTERIA AND SEMINESTED PCR TO ENHANCE DETECTION OF *SALMONELLA TYPHIMURIUM* FROM GROUND BEEF AND BOVINE CARCASS SPONGE SAMPLES

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ABSTRACT

Hydroxyapatite (HA) concentration of bacteria from ground beef and bovine carcass sponge samples was examined as a method to enhance the PCR detection of Salmonella typhimurium *in these samples. Ground beef and carcass sponge samples were inoculated with progressively lower levels of* S. typhimurium. *Concentrated (10% HA) and unconcentrated samples were prepared for PCR after 0, 2, 3, or 4 h of nonselective enrichment. Without HA concentration and enrichment,* Salmonella *in ground beef was not detected by seminested PCR, even when present at levels of 10^5 cells/mL in the 1:10 ground beef homogenates. However, when bacteria in these samples were extracted with HA, limits of detection in nonenriched samples were 10^2-10^3 CFU/mL and in enriched samples were 10^1 CFU/mL (after 2 and 3 h enrichment) and 10^0 CFU/mL (after 4 h enrichment). Without concentration or enrichment, the limit of detection of* Salmonella *in carcass sponge samples was 10^3 cells/mL. HA concentration of these samples lowered this limit to 10^0- 10^1 CFU/mL. Nine of 14 different* Salmonella *serotypes adhered to HA at proportions of 98.0% or more.*

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[2]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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INTRODUCTION

With the increasing implementation of hazard analysis and critical control point systems by the food industry, rapid microbial detection procedures are needed to expedite the surveillance and process verification requirements of meat processing establishments and other food processors (United States Department of Agriculture 1996). Conventional methods for detecting the presence of low levels of pathogenic bacteria in foods require enrichment and cultivation on selective media, followed by screening and subsequent confirmation of isolate identity from positive samples using biochemical or immunological tests. These schemes can require several days to complete. Nucleic acid probe hybridization methods and immunological assays have hastened the time required for detection of microorganisms in foods, but typically require concentrations of the target pathogen of 10^4-10^6 per mL in order to obtain a detectable signal (Blackburn *et al.* 1994; Swaminathan and Feng 1994; Tian *et al.* 1996). Enrichment incubations of several hours to a day or more are necessary to obtain adequate target cells for these assays, and total analysis time is thus increased. PCR amplification assays have the potential to overcome this limitation, but because of small sample application volume, low levels of contamination, and potential PCR-inhibitory food components, most PCR procedures for the detection of pathogens in foods still require overnight enrichment prior to amplification (Payne and Kroll 1991; Swaminathan and Feng 1994; Wilson 1997). Effective methods for separating and concentrating bacteria from foods have the potential to reduce the extra time required for the cultural enrichment of target microorganisms for use in rapid microbial detection assays (Payne and Kroll 1991). Filtration of sample suspensions and immunomagnetic separation (IMS) have been examined for use in separating and concentrating bacteria from meats, and other food and environmental samples, for subsequent PCR detection (Bej *et al.* 1991; Kapperud *et al.* 1993; Venkateswaran *et al.* 1997; Widjojoatmodjo *et al.* 1992). These methods can speed and enhance PCR detection by concentrating low levels of target bacteria into smaller sample volumes, removing target bacteria from particulates and other PCR-inhibitory substances that may be present in the sample, and in the case of IMS, by providing a purer target cell sample for PCR. In addition to shortening the time required to obtain results, efficient methods for bacterial concentration would allow for the collection of larger, more representative food samples, thereby increasing the probability of detection.

In previous work, we demonstrated the ability of crystalline hydroxyapatite (HA) to remove and concentrate bacteria from suspensions, including ground beef, feces, and bovine carcass sample suspensions (Berry and Siragusa 1997). Bacteria adhered to HA and were removed from suspensions quickly, and a number of different species of both spoilage and pathogenic foodborne bacteria adhered to HA in high percentages. The objective of the current work was to demonstrate the

effectiveness of HA concentration of bacteria to speed rapid detection, by examining the ability of HA concentration of *S. typhimurium* from ground beef and bovine carcass sponge samples to enhance PCR detection of low levels of this organism.

MATERIALS AND METHODS

Bacterial Strains, Media, and Culture Conditions

The *Salmonella* serotypes used in this study are listed in Table 1. Cultures were inoculated from frozen glycerol stocks into trypticase soy broth (BBL, Cockeysville, MD) supplemented with 0.5% yeast extract and incubated 18 h at 37C prior to use.

Salmonella were enumerated on either tryptic soy agar (Difco Laboratories, Detroit, MI) containing 0.5% yeast extract (TSA-YE) or Rambach agar (RA [Rambach 1990]; E. Merck, Gene-Trak Systems, Hopkinton, MA). Aerobic plate count (APC) determinations were made by plating samples onto TSA-YE. Samples were serially diluted if necessary in 2% buffered peptone water (BPW; BBL) and plated in duplicate by either spread plating or spiral plating (model D; Spiral Biotech, Bethesda, MD). Plates were incubated at 37C for 24 h.

HA Preparation

Sterile 0.5-mL volumes of HA (H-0252; Sigma Chemical Co., St. Louis, MO) were prepared as previously described (Berry and Siragusa 1997). HA was washed thoroughly in sterile, deionized, distilled H_2O (ddH_2O), then measured into glass test tubes to settled volumes of 0.5 mL. These fractions were washed once in 5 mL of filter-sterilized adsorption buffer (5 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 1 mM K_2HPO_4 [pH 7.2]) (Clark *et al.* 1985) and sterilized by autoclaving. The overlying buffer was removed prior to adding sample suspensions.

Determination of HA Adherence Characteristics of Different *Salmonella* Serotypes

One mL volumes of 18 h *Salmonella* cultures were pelleted by centrifugation (8,160 × *g*) and washed twice in adsorption buffer. Cells were diluted in adsorption buffer to levels of 10^5 cells/mL. Determination of HA adherence of *Salmonella* was carried out essentially as described by Berry and Siragusa (1997). Briefly, 4.5-mL volumes of the cell suspensions were added to the prepared 0.5-mL volumes of HA (10% HA [vol/vol]) and the tubes were inverted to resuspend the HA. The tubes were mixed for 5 min by gentle mixing on a rocking platform shaker, then HA was allowed to settle to the bottom of the tubes for 5 min. Cells in the initial

TABLE 1.
ADHERENCE OF *SALMONELLA* SEROTYPES TO HA

Salmonella serotype	Source [a]	Cells available for adherence (CFU/ml in control [no HA]) [b]	% Adherence (SD) [b,c]
S. arizonae	ATCC 13314	3.18×10^5	40.7 (6.8)^A
S. cerro	APHIS	7.90×10^5	98.4 (0.1)^B
S. dublin	NVSL	5.78×10^5	98.3 (0.7)^B
S. enteriditis	NVSL	8.78×10^5	99.0 (0.1)^B
S. enteriditis	ATCC 13076	1.22×10^5	79.2 (1.4)^B
S. kentucky	NVSL	5.21×10^5	99.5 (0.4)^B
S. montevideo	APHIS	6.10×10^5	99.6 (0.1)^B
S. newport	APHIS	8.70×10^5	89.5 (5.4)^B
S. typhimurium	APHIS	5.05×10^5	98.1 (0.0)^B
S. typhimurium	FSIS	2.08×10^5	91.0 (0.3)^B
S. typhimurium	NADC	5.63×10^5	98.8 (0.1)^B
S. typhimurium LT2	Lab strain	1.83×10^5	98.4 (0.1)^B
S. typhimurium	ATCC 14028	1.87×10^5	98.0 (0.1)^B
Salmonella var. 9,12: non-motile	NVSL	5.17×10^5	39.5 (26.7)^A

[a] ATCC, American Type Culture Collection; APHIS, Animal and Plant Health Inspection Service; NVSL, National Veterinary Service Laboratory; NADC, National Animal Disease Center.
[b] Values are means of duplicate determinations.
[c] Values followed by the same letter are not significantly different. ($P \leq 0.01$)

suspensions and in the supernatants following HA concentration were enumerated on TSA-YE to determine percent adherence to HA.

HA Concentration of *S. typhimurium* from Ground Beef

Three packages of fresh 80%/20% (lean/fat) ground beef were obtained from three local grocery stores. The ground beef was stored frozen at -20C, and allowed to thaw at 4C for 24 h prior to use in experiments. One package was used for each of the three replicate experiments. For each experiment, 50 g of ground beef were measured into each of six sterile beakers. Four mL of an 18 h culture of *S. typhimurium* ATCC 14028 were collected by centrifugation (8,160 × g) and washed once in sterile 0.85% NaCl (SS), then resuspended in 1 mL of SS. This volume was then serially diluted 10-fold in SS to obtain separate volumes containing from 10^8 to 10^3 cells/mL. A 0.5-mL volume from each dilution was added to separate 50 g samples of ground beef and mixed in thoroughly using a sterile wooden tongue depressor. The final cell concentrations of *S. typhimurium* in the six ground beef samples ranged from levels of 10^6 to 10^1cells/g.

Ten grams of each inoculated ground beef sample were weighed into filtered stomacher bags, 90 mL of adsorption buffer were added, and the samples were pummeled for 2 min (Stomacher 400; Tekmar, Inc., Cincinnati, OH). For control samples, 1-mL volumes of the filtered stomachates were removed to sterile microcentrifuge tubes and prepared for PCR. For HA concentration, duplicate 4.5-mL volumes of the filtered stomachates were added to 0.5-mL HA and extracted as described above. Following removal of the supernatants, one duplicate HA fraction from each sample was prepared for PCR amplification. One mL of BPW was added to each of the remaining duplicate fractions. The HA was resuspended in the BPW to mix, and the tubes were incubated at 37C in a shaker incubator (50 rpm) for 2, 3, or 4 h. Following incubation, HA was allowed to settle for 5 min, the BPW supernatant was removed, and the remaining HA was prepared for PCR amplification. The initial 1:10 ground beef stomachates comprised the unconcentrated enriched control samples and were incubated at 37C with shaking at 50 rpm for 2, 3, or 4 h. Following incubation, 1-mL volumes of the filtered stomachates were removed and prepared for PCR.

At the initial sampling time, *Salmonella* in the ground beef stomachates and in the HA supernatants were enumerated by plating onto RA to determine the extent of HA concentration of *Salmonella* from the sample suspensions. In addition, the APC of the uninoculated ground beef samples were determined.

HA Concentration of *S. typhimurium* from Bovine Carcass Sponge Samples

For each of the three replicate experiments, ten chilled beef carcasses were sponge sampled at 24 h postslaughter at a local cow/bull processing facility as outlined in the Food Safety and Inspection Service sample collection guidelines for isolation of *Salmonella* from bovine carcasses (United States Department of Agriculture 1996). The samples were a composite of three 100-cm^2 areas from the brisket, rump, and shoulder of the carcass, using 25 mL of BPW as the sponge solution. The samples were transported immediately to the laboratory at the Roman L. Hruska U.S. Meat Animal Research Center. An additional 25-mL volume of BPW was added to each sponge sample, and the contents were stomached for 1 min. All sponge stomachates were then pooled in a sterile flask and mixed, and 27-mL aliquots of the pooled stomachates were dispensed to sterile tubes. Two mL of an 18 h culture of *S. typhimurium* ATCC 14028 were collected (8,160 × g) and washed once in BPW, then resuspended in 1 mL of BPW. This volume was diluted further in additional pooled stomachate to a level of 10^6 cells/mL. This inoculum was then serially diluted 10-fold in the 27-mL aliquots of pooled stomachates to yield six bovine carcass sponge samples containing *S. typhimurium* ranging in levels of 10^5 to 10^0 cells/mL.

For unconcentrated control samples, 1-mL volumes of the six prepared sponge samples were removed to sterile microcentrifuge tubes and prepared for PCR. For

HA concentration, duplicate 4.5-mL volumes of the same six samples were extracted with 0.5-mL HA. Following removal of the HA supernatants, one duplicate HA fraction from each sample was prepared for PCR amplification. As described for ground beef samples above, one mL of BPW was added to each of the remaining duplicate fractions and HA was resuspended in the BPW to mix. The duplicate HA fractions containing BPW and remaining inoculated pooled sponge samples (unconcentrated enriched controls) were incubated at 37C with shaking for 2, 3, or 4 h. Following incubation, these concentrated and unconcentrated samples were prepared for PCR.

As described for ground beef samples, at the initial sampling time *Salmonella* levels in the inoculated pooled sponge samples and in the HA supernatants following concentration were determined by plating on RA. The APC of the uninoculated pooled sponge stomachates were also determined.

Determination of Indigenous *Salmonella* in Ground Beef and Bovine Carcass Sponge Samples

All ground beef samples and pooled sponge stomachates were sampled prior to inoculation to determine the presence of indigenous *Salmonella*. Uninoculated portions of the samples were diluted 1:10 in BPW and enriched at 37C for 18 h. Subsequent selective enrichments were performed as recommended by bioMérieux Vitek, Inc. (Hazelwood, MO) for the detection of *Salmonella* from raw meats using the VIDAS *Salmonella* (SLM) Assay and the automated miniVIDAS instrument.

Sample Preparation for PCR

Using sterile transfer pipets, the HA fractions from concentration experiments were removed to siliconized microcentrifuge tubes, and pelleted by a brief pulsing in a microcentrifuge (82 × g, 2 s). The overlying buffer was removed, then 500 µL of ddH_20 and 50 µL of 0.15 M sodium phosphate buffer (SPB; pH 6.8) were added and mixed in by pipetting and by inverting the tubes to resuspend the HA. The samples were then heated for 10 min in a boiling water bath. Following brief cooling on ice, the HA fractions were pulsed in a microcentrifuge as described above, the supernatant removed, and the HA fractions washed twice with 500 µL of 1.0 M NaCl, with thorough mixing of the HA and fluid volume accomplished each time by pipetting and by inverting the tubes. Following removal of the final NaCl wash, DNA adsorbed to the HA was eluted by washing the HA twice with 500 µL of 60C 0.5 M SPB (pH 6.8). DNA in the elution buffer was desalted using Microcon-100 microconcentrators (Amicon, Inc., Beverly, MA) and washed with 500 µL of ddH_20. The desalted concentrates were brought to final volumes of 50-100 µL with ddH_20 and frozen at -20C until use in PCR.

Unconcentrated control samples (both enriched and nonenriched) were pulsed briefly in a microcentrifuge, then the supernatant was transferred to a fresh

microcentrifuge tube. These samples were heated for 10 min in a boiling H_2O bath and frozen at −20C for PCR amplification.

Seminested PCR Amplification

PCR amplification mixtures were composed of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the four deoxynucleotide triphosphates, and 0.5 μM of each primer. *Taq* DNA polymerase was obtained from Life Technologies, Inc. (Gaithersburg, MD) and 1.25 U were used for each 50 μL reaction. The sequences of the oligonucleotide primers used in the first-step PCR were 5′-TGCCTACAAGCATGAAATGG-3′ and 5′-AAACTGGACCACGGTGACAA-3′; these primers have been examined for use in *Salmonella* detection (Stone *et al.* 1994, 1995a,b) and amplify a 457-bp region of the *invE* and *invA* genes of *S. typhimurium* (Galán *et al.* 1992; Ginocchio *et al.* 1992). For the second-step PCR, the forward primer utilized in the first step was used with a reverse primer of sequence 5′-GAGAAGACAACAAAACCCAC-3′, corresponding to nucleotides 356 to 337 of the *invA* gene and contained within the first-step amplicon. Five-μL volumes of the experimental sample preparations were amplified in the first-step PCR; for the second-step amplifications, 1 μL of the first-step reactions were added to 25 μL volumes (total) of the composition described above. For both steps, PCR was initiated by denaturation for 5 min at 94C, followed by 30 cycles consisting of 30 s at 94C, 30 s at 52C, and 45 s at 72C, and completed by a final extension of 5 min at 72C. PCR products (8 μL) were electrophoresed on 1.5% agarose gels in 1 × TAE buffer and visualized by ethidium bromide staining.

DNA Sequencing, Southern Blotting and Hybridization

The forward primer described above and a reverse primer of sequence 5′-GAAGACAACAAAACCCACCG-3′ (corresponding to nucleotides 354 to 335 of the *invA* gene) were used to amplify a 433-bp fragment from the *invE* and *invA* genes of *S. typhimurium* ATCC 14028, for use as a probe for confirmation of the specificity of the seminested PCR to detect *Salmonella* in the experimental samples. The expected nucleotide sequence of the 433-bp fragment was confirmed by sequencing both strands with the ABI Prism BigDye Terminator Cycle Sequencing kit and an ABI 377 DNA sequencer (PE Applied Biosystems, Foster City, CA).

The probe was biotinylated with BrightStar™ psoralen-biotin labeling kit (Ambion, Inc., Austin, TX). The electrophoresed amplified products were transferred to nylon membranes by Southern blotting (Southern 1975). The nylon membranes were prehybridized for 2 h at 68C in a solution of 5 × SSC (1 × SSC is 0.15M NaCl and 15 mM sodium citrate, pH 7.0), 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/mL of denatured, sheared salmon sperm DNA. The membranes

were hybridized for 18 h at 68C in the hybridization solution described above, containing 14 ng/mL of the biotinylated probe. Following hybridization, the membranes were washed twice with 2 × SSC -0.1% SDS for 5 min at room temperature, and once with 0.5 × SSC-0.1 % SDS for 15 min at 68C. Detection of the hybridized probe was performed as described in the manufacturer's protocol (Ambion) and membranes were exposed to Hyperfilm ECL film (Amersham Life Science, Inc., Arlington Heights, IL).

Data Analysis

Analysis of variance, and the Tukey-Kramer multiple comparisons test or the unpaired t test were performed on percent adherence data using Instat Version 3.00 statistical analysis package (GraphPad Software, Inc., San Diego, CA).

RESULTS

Determination of HA Adherence Characteristics of Different *Salmonella* Serotypes

Percent HA adherence of the 14 different *Salmonella* serotypes examined is shown in Table 1. In general, most of the *Salmonella* adhered to HA at high percentages, with nine of the 14 serovars adhering at percentages of 98.0% or more. The HA adherence of two strains, *S. arizonae* and a nonmotile *Salmonella* were significantly lower, at ca. 40.0%.

Percent HA Adherence of *S. typhimurium* from Ground Beef and Carcass Sponge Stomachates

Percent adherence values of *S. typhimurium* from both ground beef and bovine carcass sponge samples typically were greater than 95%, and ranged from 81.3 to 99.4% (Table 2). In some sample stomachates that contained very low initial levels of cells, *S. typhimurium* remaining in the supernatant following HA concentration was below detectable levels, giving an apparent 100% adherence. Adherence values were not significantly different for ground beef and sponge samples, or for the different cell levels ($P > 0.05$; apparent 100% adherence values were not included in the statistical analyses). High numbers of blue coliform colonies in two of the three bovine carcass sponge samples obscured the red *Salmonella* colonies at low inoculation levels (Rambach 1990), preventing accurate enumeration.

Specificity of Seminested PCR Primers

Positive seminested PCR reactions yielded the expected target 435-bp product. Southern hybridization of PCR products with the psoralen-biotin labeled 433-bp

TABLE 2.
ADHERENCE OF *S. TYPHIMURIUM* FROM GROUND BEEF AND BOVINE CARCASS SPONGE SUSPENSIONS

Sample	Percent adherence (cells available for adherence) [a]					
	Target CFU/ml in suspension:					
	10^5	10^4	10^3	10^2	10^1	10^0
Ground Beef 1	98.2 (1.98×10^5)	99.3 (4.39×10^4)	99.4 (4.57×10^3)	98.7 (5.19×10^2)	"100" [b] (4.75×10^1)	"100" (3.50×10^0)
2	81.8 (8.35×10^4)	98.3 (5.61×10^4)	95.2 (3.11×10^3)	99.0 (3.27×10^2)	83.0 (3.25×10^1)	"100" (1.15×10^1)
3	96.8 (4.95×10^5)	96.3 (4.40×10^4)	98.3 (5.77×10^3)	96.9 (3.38×10^2)	95.9 (4.85×10^1)	"100" (1.55×10^1)
Sponge 1	81.3 (2.57×10^5)	94.4 (2.69×10^4)	93.9 (2.49×10^3)	82.3 (1.13×10^2)	ND[c]	ND
2	95.1 (1.82×10^5)	93.9 (1.96×10^4)	91.7 (1.32×10^3)	84.8 (1.78×10^2)	85.8 (1.27×10^2)	ND
3	97.6 (3.48×10^5)	97.6 (3.66×10^4)	97.5 (4.09×10^3)	98.4 (3.22×10^2)	96.4 (2.76×10^1)	"100" (2.5×10^0)

[a] Values are means of duplicate determinations.
[b] "100," cells remaining in the supernatant following HA concentration were below detectable levels.
[c] ND, not determined; high levels of coliforms in these samples prevented accurate *Salmonella* enumeration.

fragment from the *invE* and *invA* genes of *S. typhimurium* ATCC 14028 confirmed the specificity of this seminested PCR (Fig. 1).

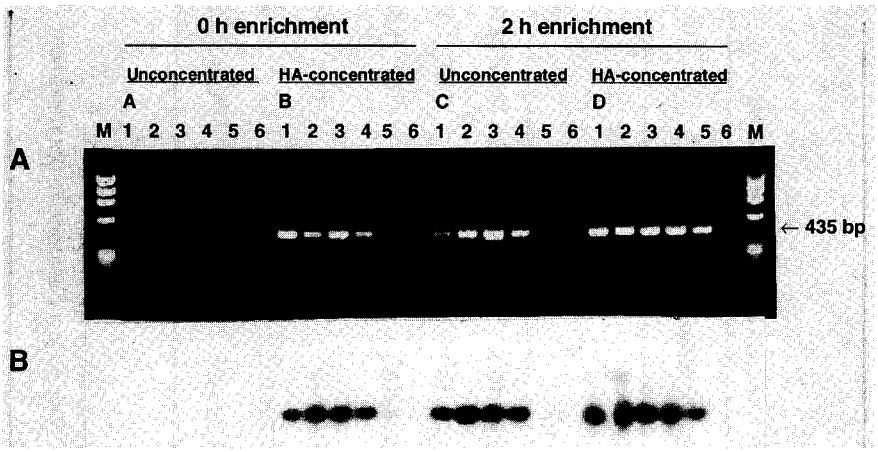

FIG. 1. GEL ELECTROPHORESIS AND HYBRIDIZATION ANALYSIS OF PCR PRODUCTS FROM 1:10 GROUND BEEF HOMOGENATES

The 1:10 ground beef homogenates contained initial levels of 10^5-10^0 CFU/mL *S. typhimurium*. Shown are PCR products from samples taken at initial sampling time (t = 0 h) and after 2 h of enrichment (t = 2 h), and with or without HA concentration. (A) Amplified products were electrophoresed and visualized by ethidium bromide staining. Lanes M contain ϕX174 Hae III digest; lanes A1-6 contain 10^5-10^0 CFU/mL *S. typhimurium*, t = 0 h, unconcentrated; lanes B1-6 contain 10^5-10^0 CFU/mL *S. typhimurium*, t = 0 h, HA-concentrated; lanes C1-6 contain 10^5-10^0 CFU/mL *S. typhimurium*, t = 2 h, unconcentrated; lanes D1-6 contain 10^5-10^0 CFU/mL *S. typhimurium*, t = 2 h, HA-concentrated. (B) Corresponding Southern blotting and hybridization with 433-bp psoralen-biotin labeled probe.

Seminested PCR Detection of HA-Concentrated *S. typhimurium* from Ground Beef and Carcass Sponge Samples

Results are summarized in Table 3. Without HA concentration and enrichment, *S. typhimurium* in ground beef was not detected by seminested PCR and agarose gel electrophoresis, even when present at levels up to 10^5 cells/mL in the 1:10 diluted ground beef stomachates (10^6 cells/g in ground beef). Concentration of *S. typhimurium* from these unenriched samples with HA improved the sensitivity of detection by at least 1000-10,000-fold, allowing detection in samples containing 10^2-10^3 CFU/mL. Short enrichment incubation periods of 2-4 h lowered the limits of detection for both unconcentrated and HA-concentrated samples (Table 3); however, in HA-concentrated samples, *S. typhimurium* detection limits were lower, at 10^1 CFU/mL after 2 and 3 h of enrichment and 10^0 CFU/mL after 4 h of enrichment. Similar results were seen for *Salmonella* detection in bovine carcass sponge samples (Table 3). *S. typhimurium* could be detected in sponge samples

TABLE 3.
SEMINESTED PCR DETECTION OF *SALMONELLA* IN GROUND BEEF AND BOVINE CARCASS SPONGE SAMPLE SUSPENSIONS[a]

Sample	Before enrichment (t = 0 h)												Enrichment Time (h)	After enrichment for 2, 3 or 4 h											
	Unconcentrated						HA concentrated							Unconcentrated						HA concentrated					
	Target CFU/ml in initial suspension:													Target CFU/ml in initial suspension:											
	10^5	10^4	10^3	10^2	10^1	10^0	10^5	10^4	10^3	10^2	10^1	10^0		10^5	10^4	10^3	10^2	10^1	10^0	10^5	10^4	10^3	10^2	10^1	10^0
Ground Beef 1	–	–	–	–	–	–	+	+	+	+	–	–	2	+	+	+	+	–	–	+	+	+	+	+	–
2	–	–	–	–	–	–	+	+	+	–	–	–	3	+	–	–	–	–	–	+	+	+	+	+	–
3	–	–	–	–	–	–	+	+	+	+	–	–	4	+	+	+	–	–	–	+	+	+	+	+	+
Sponge 1	+	+	+	–	–	–	+	+	+	+	+	+	2	+	+	+	+	+	–	+	+	+	+	+	+
2	+	+	+	–	–	–	+	+	+	+	+	–	3	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	–	–	–	+	+	+	+	+	–	4	+	+	+	+	+	+	+	+	+	+	+	+

[a] +, Positive PCR amplification; –, negative PCR amplification.

containing 10^3 CFU/mL without enrichment or HA concentration. HA concentration of these nonenriched samples further lowered the detection limit to 10^0- 10^1 CFU/mL. Because of the greater sensitivity of the seminested PCR in the sponge samples, there was little difference in the detection limits after the short enrichment incubations, although for the samples enriched for 2 h, HA concentration lowered the detection limit from 10^1 to 10^0 CFU/mL. No indigenous *Salmonella* were recovered from uninoculated ground beef or bovine carcass sponge samples.

DISCUSSION

In this study, the ability of HA concentration to remove and concentrate bacteria from ground beef and bovine carcass sponge samples was demonstrated, and the utility of this procedure to subsequently enhance PCR detection of low levels of *Salmonella* in these samples was shown.

HA concentration is robust; a 10% level of HA consistently removed high percentages of contaminating *Salmonella* from a large sample volume in the presence of high levels of fat, blood, and meat particles, without the necessity of prior centrifugation or filtration to clarify the samples. Also, HA concentration efficiency was similar when either high or low concentrations of *Salmonella* were present in the ground beef and sponge samples. Previous work suggests that levels of HA lower than 10% possibly may be used; 2.5% HA was able to remove 93.8% of an initial level of 10^6 CFU/mL *Escherichia coli* from a buffer suspension (Berry and Siragusa 1997). In addition, HA concentration of *Salmonella* is rapid, taking only 5 min, and requires no special equipment. This simplicity, rapidity, and ease of use indicates HA concentration would be amenable for field use.

Homogenates of 1:10 dilutions of ground beef are more complex samples than are carcass sponge samples, in terms of total organic load. It is for this reason that HA concentration had greater impact on *Salmonella* detection in ground beef samples than sponge samples. Without HA concentration, *Salmonella* could not be detected in any of the unenriched ground beef homogenates, even at the highest level of inoculation (10^5 CFU/mL in 1:10 homogenate, 10^6 CFU/g in ground beef). The short enrichment incubations enhanced the sensitivity of PCR detection for both unconcentrated and concentrated ground beef and sponge samples, but this relative increase in sensitivity was greater for the ground beef samples (Table 3). Cultural enrichment typically has been necessary to allow PCR detection of pathogenic bacteria in ground beef samples, and incubation times ranging from 6 to 24 h or more are reported to be necessary for detection of very low cell levels, depending upon the sample preparation, DNA extraction procedure, PCR protocol, and PCR product detection method employed (Aabo *et al.* 1995; Chen *et al.* 1998; Gannon *et al.* 1992; Soumet *et al.* 1997; Uyttendaele *et al.* 1998; Venkateswaran

et al. 1997; Witham *et al.* 1996). Uyttendaele *et al.* (1998) examined the effect of extended refrigerated storage on PCR detection of enterohemorrhagic *E. coli* (EHEC), and recommended 24 h of cultural enrichment for reliable detection of low numbers of EHEC. While enrichment time does extend the time required for detection, it also can provide greater confidence that only viable cells are being detected (Stone *et al.* 1995a; Witham *et al.* 1996). In addition, more target DNA template may be provided by nonselective enrichment incubations that are long enough to allow cells to initiate rapid growth. Rapidly growing cells contain more DNA per cell than do cells that are doubling at rates of 1 h or more (Bremer and Dennis 1987), and this may further lower the threshold of detection.

Another particular advantage of HA concentration is its nonspecific nature. It previously was demonstrated that a variety of foodborne bacterial species will adhere to HA in high proportions (Berry and Siragusa 1997), so HA concentration could easily be incorporated into existing PCR protocols for other bacteria, as described here. However, the determination of HA adherence characteristics of a number of strains of the species of interest is recommended, as this work and previous research has shown that not all bacteria may adhere to HA in high numbers (Berry and Siragusa 1997). A nucleic acid-based rapid assay method was chosen to demonstrate the use of HA concentration for two reasons. First, while bacteria can be consistently and quantitatively concentrated by adherence to HA, to date we have not accomplished the quantitative removal of bacteria from this compound. Second, the application of HA for use in nucleic acid isolation has been well demonstrated, so established methods for eluting bacterial DNA could be used (Britten *et al.* 1969; Johnson 1994). Laborious DNA extraction techniques were avoided by boiling the HA fraction containing the bound cells to extract the DNA, then eluting the DNA from HA using 0.5 M phosphate buffer (60C). In addition to concentrating bacteria from the samples, it is likely that the use of HA as described here contributed to the improved detection by aiding in the recovery of a purer DNA sample.

The oligonucleotide primers used in the first step of the seminested PCR amplification have been examined extensively by Stone *et al.* (1994, 1995a,b) for use in *Salmonella* detection in veterinary clinical samples. In their examination of the use of this PCR protocol to detect *S. typhimurium* from rectal swabs of experimentally infected beagles, Stone *et al.* (1995b) found that a 429-bp nonspecific product was amplified by this primer pair in samples that had been enriched for 24 h. The sequencing of this nonspecific amplicon revealed sequence similarity to the *Escherichia coli* aldehyde dehydrogenase gene (Heim and Strehler 1991), and little similarity to internal sequences of the 457-bp *Salmonella* target fragment (Stone *et al.* 1995b). To increase the specificity of the PCR for the current study, a second reverse primer was selected from internal sequences of the 457-bp specific fragment and used in the second-step of a seminested PCR. This primer combination amplified a 435-bp fragment within the first-step amplicon.

A significant advantage of nested PCR protocols includes their high specificity, which may make lengthy confirmational probe hybridization procedures unnecessary (Kaneko *et al.* 1989; Persing 1993). For the current work, the specificity of the second-step 435-bp amplicon was confirmed by hybridization; further work in other sample types will be necessary to determine if the specificity of these primer pairs is adequate to omit confirmational Southern blot hybridization.

This study describes a HA concentration-seminested PCR method that is sensitive and specific for detecting *Salmonella* in ground beef and bovine carcass sponge samples. HA concentration of the bacteria enhanced the PCR detection for both nonenriched and enriched samples. With HA concentration, *S. typhimurium* initially present at 10^0 CFU/mL in the 1:10 ground beef homogenates could be detected after 4 h of enrichment. Without enrichment, 10^0-10^1 *Salmonella*/mL could be detected in HA-concentrated carcass sponge samples. HA concentration of bacteria is both simple and fast, and for these reasons would be useful not only for routine laboratory analysis but for field work, and easily incorporated into an automated system. Finally, because bacterial adherence to HA is nonspecific and nucleic acid can be readily eluted from HA, this HA concentration method may easily be adapted to existing PCR protocols for other bacteria, or to other nucleic acid-based detection methods.

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