Broad-Range (Pan) Salmonella and Salmonella Serotype Typhi–Specific Real-Time PCR Assays

Potential Tools for the Clinical Microbiologist

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Abstract

We describe broad-range salmonellae (ie, Salmonella) and Salmonella serotype Typhi–specific LightCycler (Roche Diagnostics, Indianapolis, IN) real-time polymerase chain reaction assays. We validated these with a battery of 280 bacteria, 108 of which were salmonellae representing 20 serotypes. In addition, 298 isolates from 170 clinical specimens that were suspected to possibly represent salmonellae were tested with the pan-Salmonella assay. Finally, the pan-Salmonella assay also was used to test DNA extracts from 101 archived, frozen stool specimens, 55 of which were culture-positive for salmonellae. Both assays were 100% sensitive and specific when cultured isolates of the battery were tested. The pan-Salmonella assay also characterized correctly all salmonellae on the primary isolation agar and was 96% sensitive (53/55) and 96% specific (49/51) when nucleic acid extracts from direct stool specimens were tested. These assays represent potential tools the clinical microbiologist could use to screen suspect isolates or stool specimens for Salmonella.

Salmonella species are important causes of enteritis throughout the world. In addition, Salmonella enterica serotypes Typhi and Paratyphi are important causes of enteric fever in underdeveloped countries that lack adequate sewage disposal and water treatment facilities.1,2 It is standard practice in many clinical microbiology laboratories to culture for Salmonella and Shigella species by using primary isolation media, such as MacConkey and Hektoen Enteric agars.3,4 Isolates suspected to possibly represent salmonellae (ie, lactose non-fermenting colonies with or without hydrogen sulfide production) are submitted for additional screening or identification, depending on individual laboratory practices.5-7 These practices, although effective, are time-consuming and costly.

The identification of Salmonella directly from the primary isolation plates or, ideally, directly from stool samples is attractive.8 This has been achieved using polymerase chain reaction (PCR) for the invA gene, a gene associated with the invasive nature of Salmonella.9 We hypothesized that the prgK gene, which is part of a genetic complex that also is thought to be important for enterocyte invasion, might serve as a useful target for a real-time PCR-based assay for the detection of Salmonella enterica from cultures and clinical stool specimens.10-12 In addition, we developed a real-time PCR assay directed against the vexC gene, which encodes for the Vi antigen, as a means of detecting the Salmonella serotype Typhi. The usefulness of the gene that encodes for the Vi antigen as a specific target for the identification of Salmonella Typhi has been demonstrated previously.13-15 The real-time PCR assays described were validated against a large battery of lysates from well-characterized bacteria, including 108 strains of Salmonella, representing 20 serotypes. In addition, we used...
Materials and Methods

Primer and Probe Design

The primers and fluorescent resonance energy transfer (FRET) probes, for use with LightCycler system (Roche Diagnostics, Indianapolis, IN), for the broad-range or pan-Salmonella assay were designed based on the prgK gene of Salmonella serotype Typhimurium (GenBank accession number, AE008831).10,16 This primer set generated a 193-base-pair nucleotide product that demonstrated high predicted specificity for Salmonella when submitted to a National Center for Biotechnology Information (NCBI) Blast, with perfect matches obtained only for Salmonella enterica. The GenBank accession numbers of these perfect matches were AE008831, L33855, AC117230, and AL627276.

The PanSalm FRET hybridization probe set was designed to be 100% homologous for Salmonella Typhimurium. The 3′ end of the donor FRET hybridization probe (PanSalmHP1) was labeled with fluorescein (fam). The 3′ end of the acceptor FRET hybridization probe (PanSalmHP2) was phosphorylated (p) to prevent probe extension during PCR, and the 5′ end was labeled with 705-N-hydroxysuccinimide ester (LC Red 705). The sequences for the pan-Salmonella primers and probes were as follows: PanSalmF, 5′-CTTCTTATTGTGCGGCA-3′ (forward primer; position 4179-4196); PanSalmR, 5′-GCGATGTGATTTGACGCATTCG-3′ (reverse primer; position 42610-42595); PanSalmHP1, 5′-GGATGTGATTGTATTGGCGTGATG-[fam]-3′ (donor FRET probe; position 4266-4235); and PanSalmHP2, 5′-[LC Red 705]-CTGGTTCGGCAAAGACGCACG-p-3′ (acceptor FRET probe; position 42477-42458).

PCR Reagents and Thermocycling Parameters

LightCycler FastStart DNA Master Hybridization Probe kit reagents (Roche Diagnostics), primers, and probes were combined in a master mix. The PCR reaction was carried out in LightCycler capillary tubes (Roche Diagnostics) with 15 µL of master mix and 5 µL of sample. For the pan-Salmonella PCR assay, the following concentrations were used: 1.0 µmol/L of both forward and reverse primers, 0.4 µmol/L of both PanSalm FRET hybridization probes, and 4.0 mmol/L of magnesium chloride. The same concentrations were used for the Salmonella Typhi–specific assay, except a 3.0-mmol/L concentration of magnesium chloride was used. The thermocycling protocol of the pan-Salmonella PCR consisted of 10 minutes at 95°C for DNA polymerase activation, 45 cycles of PCR amplification (95°C for 10 seconds, 50°C for 10 seconds, and 72°C for 20 seconds), postamplification melting (35°C to 95°C at 0.1°C/second), and a cooling step (35°C for 30 seconds). The protocol for the Salmonella Typhi–specific assay was the same, except primer annealing occurred at 55°C for 10 seconds and the postamplification melting range from 45°C to 75°C.

Validation Battery

The sensitivity and specificity of both assays were assessed using lysates of 280 well-characterized bacterial strains obtained from Duke University Medical Center (Durham, NC) and the Cleveland Clinic Foundation (Cleveland, OH) stock collections of bacteria. These bacteria included numerous American Type Culture Collection (Manassas, VA) strains. The other non-Salmonella stock isolates were identified by traditional biochemical methods by experienced microbiologists and were characterized unambiguously. Of the 280 isolates tested, 108 were Salmonella species, which included 23 Salmonella Typhi and 24 Salmonella Paratyphi. The 108 Salmonella isolates tested consisted of 20 serotypes, including those routinely identified (ie, commonly occurring) in these 2 clinical microbiology laboratories. The serotyping of these isolates was performed in the local public health laboratories according to routine. A variety of commonly encountered bacteria was included in the remaining 172 non-Salmonella bacteria tested and included
other enteric pathogens such as Shigella species, Yersinia enterocolitica, and Escherichia coli O157:H7. 

DNA lysates of the cultured bacteria were prepared by placing a 1.0-μL loop of each cultured isolate into 500 μL of lysis buffer, which has been described previously and contains 1× tris(hydroxymethyl)aminomethane-EDTA buffer supplemented with 1% Triton X-100 (Sigma, St Louis, MO) and 0.5% polysorbate 20. This solution was heated to 100°C for 10 minutes, centrifuged at 10,000 g for 30 seconds, and stored at –20°C. The criterion for a positive test result was a cycle threshold (C_T) (ie, crossing point) of 35 cycles or fewer.

**Limits of Detection**

The limit of detection was determined only for the pan-Salmonella assay because it was not our intention to use the Salmonella Typhi–specific assay on direct specimens, but rather on cultured isolates wherein the target copy number is excessive. To determine the analytic sensitivity of this assay for lysates of Salmonella in pure culture, duplicate serial dilutions of a 0.5-McFarland suspension of Salmonella Typhimurium (American Type Culture Collection, 23854) were prepared in 0.85% saline. Lysates of each serial dilution were tested by the LightCycler *prgK* real-time PCR protocol as described. One hundred microliters from each of the serial dilutions was inoculated and spread onto sheep blood agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD) and then incubated in atmospheric oxygen at 35°C for 24 hours to determine colony counts for each dilution. The lowest limit of detection was determined by averaging the values of the duplicate quantitative cultures that were correlated with the lowest limit of detection by PCR.

A stool specimen, proven by culture to be negative for all bacterial enteric pathogens, was used to determine the analytic sensitivity of the pan-Salmonella assay for stool specimen extracts. Aliquots of this stool specimen were spiked with serial 10-fold dilutions of a 0.5-McFarland preparation of Salmonella Typhimurium, as described in the preceding text. DNA extracts were prepared, as described subsequently, for the clinical stool specimens, and real-time PCR was performed on these extracts and compared with the results of the quantitative cultures, which were performed as described in an earlier section.

### Testing Suspect Colonies From Routine Stool Cultures With the Pan-Salmonella Assay

A prospective validation of the pan-Salmonella assay was conducted during a 5-month period on all isolates from stool cultures that were suspected to possibly represent Salmonella because of morphologic features of their colonies (ie, green/green or black colonies on Hektoen enteric agar). Cell lysates were prepared as described in the preceding text from 298 suspect isolates from the stool cultures of 170 patients. The lysates were batch tested once a week by the protocol described herein, with the same positive test criterion (ie, C_T ≤35 cycles).

<table>
<thead>
<tr>
<th>Bacteria Tested</th>
<th>Pan-Salmonella</th>
<th>Serotype Typhi-Specific</th>
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</thead>
<tbody>
<tr>
<td><strong>Gram-positive bacteria tested (n = 40)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus (6), Staphylococcus epidermidis (3), Staphylococcus saprophyticus (2), Micrococcus species (2), Stomatococcus species (2), Lactobacillus species (2), Enterococcus species (3), Viridans streptococci (3), Streptococcus pneumoniae (3), group A streptococcus (3), group B streptococcus (3), Aerococcus species (3), <em>Listeria</em> species (3), <em>Bacillus</em> species (3)</td>
<td>All negative</td>
<td>All negative</td>
</tr>
<tr>
<td>Non–Salmonella gram-negative bacteria tested (n = 132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella Typhi</em> (23)</td>
<td>All positive</td>
<td>All positive</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction.

Table 1

**Validation Battery of Cultured Bacteria**

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Each batch included a DNA extract from *Salmonella Typhimurium* as a positive control sample and lysis buffer as a negative control sample. The assay results subsequently were compared with the culture results to determine the sensitivity and specificity of the assay in this setting. The *Salmonella Typhi–specific* PCR was not performed on these colony extracts, primarily because of the low incidence of *Salmonella Typhi* in the United States and because the sensitivity and specificity of the *Salmonella Typhi–specific* assay was defined previously in the validation phase of this study.

DNA Extracts From Clinical Stool Specimens

DNA extracts of 101 stool samples identified by culture or *Campylobacter* enzyme immunoassay to be positive for a bacterial enteric pathogen and 5 samples that were negative for enteric pathogens were tested. The pathogens detected by culture were *Salmonella* (55), *Shigella* (20), *E coli* O157:H7 (9), *Campylobacter jejuni* (4), *Y enterocolitica* (3), and *Plesiomonas shigelloides* (1). Nine stool samples were positive for *Campylobacter* by the ProSpecT (Alexon-Trend, Ramsey, MN) *Campylobacter* enzyme immunoassay. All stool samples were stored at –20°C before extraction. Nucleic acid extracts were prepared from thawed stool specimens with the Qiagen Stool Extraction Kit (Qiagen, Valencia, CA). The pan-*Salmonella* real-time PCR assay was performed with the master mix and thermocycling protocol described in preceding text and with the same criteria for test positivity (ie, $C_T \leq 35$ cycles). The *Salmonella Typhi–specific* PCR also was not performed on the direct stool extracts because of the low incidence of *Salmonella Typhi* in the United States. An evaluation of the *Salmonella Typhi–specific* PCR on routinely occurring stool culture isolates and nucleic acid extracts from direct stool specimens should be performed in an area with a sufficient incidence of *Salmonella Typhi* infections to appropriately validate this assay.

**Results**

**Validation Battery**

All lysates from the 108 *Salmonella* strains included in the validation battery were positive with the pan-*Salmonella* PCR, whereas only the 23 strains of *Salmonella Typhi* were positive with the *Salmonella Typhi–specific* PCR (Table 1). Figure 1 and Figure 2. The 85 strains of *Salmonella* that were not serotype Typhi were negative with the *Salmonella Typhi–specific* PCR. The 172 lysates from non-*Salmonella* bacteria were negative with both the pan-*Salmonella* and the *Salmonella Typhi–specific* assays (Table 1). These assays both demonstrated 100% sensitivity and 100% specificity when the bacterial lysates from the validation battery were tested (Table 1). The approximate analytic sensitivity of the pan-*Salmonella* assay for cultured bacteria was 8 colony-forming units per milliliter.

**Routine Stool Culture Isolates**

All suspect colony lysates from the stool samples proven by culture to contain *Salmonella* in the prospective study were positive with the pan-*Salmonella* PCR assay. All remaining suspect colonies, determined to not represent salmonellae by
routine bacteriologic methods, were negative with the pan-
Salmonella PCR. During this study, there was 1 case of appar-
etent misinoculation of lysis buffer or a mislabeling of the test
tube that resulted in an apparent false-positive result; reevalu-
ation of the 2 colony types involved gave correct identification
with the pan-Salmonella assay. Therefore, as with the valida-
tion battery, we believe the sensitivity and specificity of the
pan-Salmonella PCR on colony lysates from colonies on the
primary recovery media were both 100%.

PCR Testing of Frozen, Archived Stool Specimens

Of the 55 stool specimens positive by culture for salmo-
nellae, 53 were positive with the pan-Salmonella PCR (ie, there
were 2 false-negative PCR results) Table 2. Of the extracts
from the 51 stool specimens that were culture negative
for salmonellae, there were 2 that tested positive in the
pan-Salmonella assay (ie, 2 false-positive results). Both were
from stool samples from which E coli O157:H7 had been cul-
tured. The sensitivity and specificity for the pan-Salmonella
assay when DNA extracts from stool specimens were tested
were 96% (53/55) and 96% (49/51), respectively (Table 2).
Low-level amplification (Ct = 44) was detected in one of the
stool extracts, but this had to be categorized as a false-negative
result because of our predefined interpretation criteria.

The testing of DNA extracts of normal stool samples
spiked with serial dilutions of Salmonella Typhimurium to
determine the limit of detection of this assay demonstrated
that PCR was positive at a dilution of 10^{-5} but not at greater
dilutions. Duplicate quantitative cultures of 100 µL of this
dilution revealed 91 organisms. Therefore, the limit of detec-
tion of this assay for Salmonella in DNA extracts of stool
specimens is estimated at approximately 910 colony-forming
units per milliliter.

Discussion

The taxonomy of Salmonella has been reviewed exten-
sively by Janda and Abbott.18 Two species of Salmonella cur-
cently are recognized, S enterica and Salmonella bongori.
Eight subgroups or subspecies have been defined using a vari-
ety of molecular methods.18-20 S bongori contains only a single
subspecies or subgroup (ie, subgroup V, bongori). S enterica
contains the remaining 7 subgroups, 2 of which contain the
clinically relevant salmonellae. Most of these are contained
within subgroup I, enterica, and are subcategorized further as
serotypes (eg, Typhi, Typhimurium, Enteritidis).21,22 Subgroup
IIIa, arizonae, contains the clinically important salmonellae
that are associated with reptiles and cause human infection.

We designed and tested and now report a sensitive and
specific LightCycler real-time PCR assay for the detection of
Salmonella and another for the detection of the Typhi serotype.

The pan-Salmonella assay was directed against a single-copy
gene, which is part of a complex that is thought to be important
for the entry of salmonellae into enterocytes.23,24 A similar
genetic target, the invA gene, previously has been shown to be
useful for the detection of salmonellae by PCR.25,26 We think
this assay potentially could be useful as a molecular means of
screening colonies from stool cultures that have characteristics
that suggest the possibility of salmonellae. We proved this
hypothesis, first by testing lysates from a large battery of well-
characterized bacteria, many of which are isolated commonly
in a clinical microbiology laboratory. The validation battery
also included 108 strains of Salmonella, representing 20
serotypes, including Typhi, Paratyphi, and commonly occur-
ing serotypes. The pan-Salmonella and the Salmonella
Typhi-specific assay correctly characterized all isolate lysates
as a Salmonella species or the Typhi serotype, respectively.

The additional studies performed examined only the pan-
Salmonella assay, which was the assay that was of greatest
interest to us for implementation into our laboratory for rou-
tine testing. Furthermore, we thought additional studies of the
Salmonella Typhi-specific PCR assay on stool culture isolates
or stool nucleic acid extracts from patients in Cleveland would
not sufficiently validate this assay further, given the low inci-
dence of Salmonella Typhi infections in this area. These stud-
ies, which are important before assay implementation, should
be performed in an area wherein Salmonella Typhi is endemic
to validate the assay.

We further examined the pan-Salmonella assay in a
prospective study wherein we examined a large number of
lysates (n = 298) from colonies suspected to possibly represent

<table>
<thead>
<tr>
<th>Table 2</th>
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<tbody>
<tr>
<td>PCR of Stool Specimen Extracts</td>
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<tr>
<td>---</td>
</tr>
<tr>
<td><strong>Total Salmonella-positive stool samples</strong></td>
</tr>
<tr>
<td><strong>Salmonella serotype Typhi</strong></td>
</tr>
<tr>
<td><strong>O antigen serogroups</strong></td>
</tr>
<tr>
<td>Group B</td>
</tr>
<tr>
<td>Group C</td>
</tr>
<tr>
<td>Group D non-Salmonella Typhi</td>
</tr>
<tr>
<td>Group E or G</td>
</tr>
<tr>
<td>Not typeable</td>
</tr>
<tr>
<td><strong>Other enteric pathogens and negative stool cultures</strong></td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
</tr>
<tr>
<td>Campylobacter by EIA</td>
</tr>
<tr>
<td>Shigella sonnei</td>
</tr>
<tr>
<td>Shigella flexneri</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
</tr>
<tr>
<td>Escherichia coli O157:H7</td>
</tr>
<tr>
<td>Plesiomonas shigeloides</td>
</tr>
<tr>
<td>No pathogen isolated</td>
</tr>
</tbody>
</table>

EIA, enzyme immunoassay; PCR, polymerase chain reaction.
Salmonella because of their features on the primary inoculation media. These 298 isolates were from the routine stool cultures of 170 patients. All colonies were categorized appropriately as Salmonella or non-Salmonella species based on the results of the pan-Salmonella assay. These results largely were expected, given the results of the testing of the validation battery. This aspect of the study, however, was done on lysates prepared by routine medical technologists and done directly from media used for primary inoculation. There was a misinoculation or a mislabeling of a lysate, which reiterates the need for good quality control with any laboratory test.

Although not performed in this study, the possibility exists of testing multiple different colonies or “sweeps” from individual plates, rather than individual colonies. The feasibility of this approach remains to be proven, but it likely is possible given the low limit of detection of this assay for lysates derived from cultured isolates. Although the Salmonella Typhi-specific assay was not tested on the colonies that grew on the primary inoculation media because of the low incidence of enteric fever in the United States, it is highly likely that this assay could be used to exclude the Typhi serotype for isolates that tested positive within the pan-Salmonella assay; this, too, however, remains speculative. This prospective validation at the stool bench in our clinical laboratory demonstrated that real-time PCR was as accurate for the differentiation of Salmonella from non-Salmonella isolates as conventional methods, but considerably faster.

Not surprisingly, the pan-Salmonella assay was considerably less sensitive (approximately 2 logs) when DNA extracts from stool specimens were tested compared with colony lysates. However, even with extracts from this complex specimen type, the assay was highly sensitive with 96% (53/55) of Salmonella-containing stool samples detected. Two of the 51 stool samples that did not contain salmonellae by culture were positive with the pan-Salmonella PCR. Both of the extracts that generated false-positive results were from cultures that grew E coli O157:H7. There was no cross-reactivity with this assay, and the E coli O157:H7 isolates tested (Figure 1), nor is there any expected based on GenBank searches. The possibility remains that salmonellae were present in these stool samples but not detected by routine culture (ie, false-negative cultures), but this remains speculative. The greater than 95% sensitivity and specificity of this assay for detecting Salmonella in extracts of stool specimens suggests that, perhaps owing to the large number of organisms typically present in stool samples from patients with Salmonella enterocolitis, this assay has adequate sensitivity for a clinical application. The complete Salmonella genome contains only 1 copy of the prgK gene, which might limit the analytic sensitivity of this assay and, thus, the applicability of this assay for the detection of Salmonella directly in the blood of patients with enteric fever, in whom the number of bacteria per milliliter would be considerably lower than in stool samples.

Initial studies (data not shown) included the use of an alternative acceptor FRET probe, the sequence of which was: 5'-[LC Red 705]-AGCAGGCTTTGGCGTCTGG-p-3' (acceptor FRET probe; position 4232-4210). This probe, however, did not detect Salmonella arizonae. This is not surprising, in retrospect, because S arizonae is in a distinctly different subgroup (IIa) from the other clinically important salmonellae (subgroup I). Unfortunately, the sequence of the prgK gene for S arizonae was not available in GenBank for electronic analysis when this assay was designed. When we discovered that S arizonae was missed by the initial version of the pan-Salmonella assay, we first demonstrated that the amplicon was generated by the primers, through gel electrophoresis, and then sequenced the amplicons of S arizonae, Salmonella Typhimurium, and Salmonella Typhi for comparison. This comparison led to the redesign of the acceptor FRET hybridization probe used in this study. The extension of the 3' of the acceptor FRET probe by 4 nucleotides (GTGG) was necessary to detect S arizonae.

The use of PCR and, more recently, real-time PCR has become commonplace in molecular microbiology and molecular pathology laboratories. This technology often is used to detect fastidious microbes or microorganisms that cannot be cultivated. Real-time applications, however, have been used for the detection of commonly cultured bacteria and their mechanisms of resistance. Examples of this include the detection of Staphylococcus aureus and the mecA gene and the detection of vancomycin-resistant enterococci.27,28 In some cases, real-time PCR has been found to be cost-competitive compared with culture.29 The detection of Salmonella and the differentiation of these organisms from the many mimics that occur in stool cultures are now possible with real-time PCR of lysates from cultured colonies or direct nucleic extracts of the stool specimens. How such technologies will be incorporated into the stool bench of the future, however, remains to be seen.

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