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Sensitive Detection and Serovar Differentiation of Typhoidal and Nontyphoidal *Salmonella enterica* Species Using 16S rRNA Gene PCR Coupled with High-Resolution Melt Analysis

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Salmonella enterica species infections are a significant public health problem causing high morbidity rates worldwide and high mortality rates in the developing world. These infections are not always rapidly diagnosed as a cause of bloodstream infections because of the limitations of blood culture, which greatly affects clinical care as a result of treatment delays. A molecular diagnostic assay that could rapidly detect and identify *S. enterica* species infections as a cause of sepsis is needed. Nine typhoidal and nontyphoidal *S. enterica* serovars were used to establish the limit of detection (LOD) of a previously published 16S rRNA gene PCR (16S PCR) in mock whole blood specimens. In addition, 16 typhoidal and nontyphoidal *S. enterica* serovars were used to evaluate the serovar differentiation capability of 16S PCR coupled with high-resolution melt analysis. The overall LOD of 16S PCR for the nine typhoidal and nontyphoidal *S. enterica* serovars analyzed was <10 colony-forming units per milliliter (CFU/mL) in mock whole blood specimens, with the lowest and highest LOD at <1 CFU/mL and 9 CFU/mL, respectively. By high-resolution melt analysis, the typhoidal and nontyphoidal *S. enterica* serovar groups analyzed each generated a unique grouping code, allowing for serovar-level identification. 16S PCR coupled with high-resolution melt analysis could be a useful molecular diagnostic that could enhance the current diagnostic, treatment, and surveillance methods of *S. enterica* bloodstream infections. *(J Mol Diagn 2014, 16: 261—266; http://dx.doi.org/10.1016/j.jmoldx.2013.10.011)*

Each year in the United States, approximately 1 million domestically acquired foodborne illnesses and >350 deaths occur as a result of nontyphoidal *Salmonella enterica* species infections,[1,2] whereas worldwide, approximately 93.8 million illnesses and 155,000 deaths occur.[2–4] Typhoidal *S. enterica* species infections cause approximately 21.7 million cases of typhoid fever and >200,000 deaths annually worldwide.[3,5–7] Typhoidal and nontyphoidal *S. enterica* species infections are a significant public health problem, causing high worldwide morbidity rates and high mortality rates in the developing world.[1–7]

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Although *S. enterica* species infections are seen as a significant public health problem, the global burden of these infections is poorly characterized due, in part, to insufficient diagnostic and surveillance methods and emergence of antimicrobial-resistant *S. enterica* species.[1−12] The Centers for Disease Control and Prevention and the World Health Organization have established laboratory-based surveillance programs and guidelines for the detection, identification, treatment, and prevention of *S. enterica* species infections.[1−4,9,10,12−15] However, not all *S. enterica* species infections are properly diagnosed, leading to delays in adequate treatment and accurate surveillance data, further complicating *S. enterica* burden.[1,3−6,8,12]

The diagnosis of an *S. enterica* species infection requires isolation of the organism from feces, blood, or other sterile body fluid in a clinical laboratory.[3,6,8,12] Hospitals or other clinical settings isolating *Salmonella* from clinical specimens are required to send isolates to a public health laboratory for further identification via serotyping.[2,9,12] Overall, the organism identification process can take several days, negatively affecting timely clinical treatment decisions, as well as surveillance data.[8,12] There are reports of molecular methods that have been developed to detect *S. enterica* species infections.[16−23] However, there are no rapid identification methods for the diagnosis of *S. enterica* species from human clinical specimens approved by the US Food and Drug Administration.[3,6,8,12]

There is a need to develop a sensitive and specific assay for rapid detection and identification of *S. enterica* species infections; such an assay would greatly enhance the current diagnostic, treatment, and surveillance methods associated with this global public health problem. Use of a molecular diagnostic for rapid and accurate detection and identification of *S. enterica* species in a clinical matrix would be an ideal adjunctive to the gold standard of microbiological culture. A rapid molecular diagnostic that could accurately detect and differentiate *S. enterica* serovars would aid patient care, especially in cases of bloodstream infections. Emergency care decision making, and improved clinical outcomes by increasing timeliness to appropriate treatment and accurate surveillance data, would also be aided by improved diagnostics.

The purpose of this study was to determine the limit of detection (LOD) in mock whole blood specimens and the serovar differentiation capability of a broad-based 16S rRNA gene PCR (16S PCR) coupled with high-resolution melt analysis (HRMA) for typhoidal and nontyphoidal *S. enterica* species.

Materials and Methods

Salmonella Species

Nine typhoidal and nontyphoidal *S. enterica* serovars (Table 1), obtained from the Center for Vaccine Development (CVD; University of Maryland School of Medicine, Baltimore, MD), were used to establish the LOD of a previously described broad-based 16S PCR.[24,25] Each *S. enterica* serovar was grown in Trypticase Soy Broth (TSB; Becton Dickinson, Sparks, MD) at 37°C with continuous mixing on a platform rocker for 12 to 16 hours. At the end of the incubation period, the concentration of each *S. enterica* serovar was estimated to be approximately 10^8 colony-forming units per milliliter (CFU/mL) in TSB using McFarland Equivalence Turbidity Standard (Remel; Thermo Fisher Scientific, Lenexa, KS).

Sixteen typhoidal and nontyphoidal *S. enterica* serovars (Table 2), composed of nine groups of species, obtained from CVD were used to evaluate the serovar differentiation capability of the previously described broad-based 16S PCR coupled with HRMA.[26−28] Each *S. enterica* serovar was grown in TSB at 37°C with continuous mixing on a platform rocker for 12 to16 hours.

All serovars acquired from CVD were well-characterized clinical isolates that were established to be members of their respective serovars (eg, Typhi and Paratyphi A to C) and nontyphoidal serovars (eg, Typhimurium and Enteritidis). These organisms were chosen for analysis because of availability and because they were representative of different types of *S. enterica* that are commonly observed clinically.

Serial Dilutions and Mock Whole Blood Specimens

Ten 10-fold serial dilutions of each 10^8 CFU/mL *S. enterica* serovar stock organism were generated using 3 mL of autoclaved, filtered (Corning Inc., Corning, NY) nuclease-free water (Ambion, Life Technologies, Grand Island, NY). Mock whole blood specimens were generated from each 10-fold serial dilution by the addition of 5 mL of human whole blood [human whole blood K_2EDTA, unspun and tested negative (hepatitis B surface antigen, rapid plasma reagin, antibodies to HIV and hepatitis C virus, nonreactive HIV-1 and hepatitis C virus RNA)] (Biological Specialty Corporation, Colmar, PA). Each mock whole blood specimen ($n = 90$) was made to a total volume of 8 mL, of which 5 mL was used for nucleic acid extraction.

Titer Determination

For each *S. enterica* serovar, 100 μL of each mock whole blood specimen, ranging from $1{:}10^6$ to $1{:}10^9$ dilution, was

Table 1 LOD of 16S PCR for *S. enterica* Species Detection

Species	Serovar	LOD (CFU/mL)
Typhoidal		
S. enterica serovar Typhi	CVD A19	9
S. enterica serovar Paratyphi A	EAR 6473	9
S. enterica serovar Paratyphi B	VSM 6217	5
S. enterica serovar Paratyphi C	CVD P53	<1
Nontyphoidal		
S. enterica serovar Typhimurium	CVD A13	4
S. enterica serovar Enteritidis	CVD J73	2
S. enterica serovar Dublin	CVD R17	1
S. enterica serovar Choleraesuis variant Kunzendorf	P159	<1
S. enterica serovar Newport	361	<1

Table 2 Unique Grouping Codes Generated by HRMA for *S. enterica* Species

Organism	Grouping codes		
	V1	V3	V6
Typhoidal			
S. enterica Typhi (2)	d	a	d
S. enterica Paratyphi A (2)	k	e	l
S. enterica Paratyphi B (2)	d	e	l
S. enterica Paratyphi C	k	a	l
Nontyphoidal			
S. enterica Typhimurium (2)	d	b	d
S. enterica Enteritidis (2)	a	a	g
S. enterica Dublin (2)	k	e	h
S. enterica Choleraesuis (2)	k	a	i
S. enterica Newport	d	e	m

Parentheses indicate serovars evaluated for each *S. enterica* species.

plated to determine colony count on Trypticase Soy Agar with 5% Sheep Blood (TSA blood agar plate; Becton Dickinson, Sparks, MD) and incubated overnight at 37°C. For each *S. enterica* serovar, 1000 μL of the $1:10^{10}$ mock whole blood specimen dilution was plated onto 10 different TSA blood agar plates, in 100-μL volumes, and incubated overnight at 37°C. These 14 TSA blood agar plates were used to determine titer in terms of CFU/mL for each *S. enterica* serovar. The titer obtained for each *S. enterica* serovar was used to determine the LOD of 16S PCR in terms of CFU/mL.

Nucleic Acid Extraction

Nucleic acid was extracted from the mock whole blood specimens generated for each *S. enterica* serovar using Molzym's MolYsis Basic 5, 5 mL whole blood (Molzym GmbH and Co KG, Bremen, Germany), according to the manufacturer's instructions, with the following modifications that were in combination with our in-house nucleic acid extraction method.[24–26] Bacterial cells were harvested with centrifugation of 2100 × *g* for 20 minutes. Bacterial cell pellets were resuspended with 100 μL of autoclaved, filtered, and DNase I (Ambion, Life Technologies) treated nuclease-free water. This was followed by the addition of 60 μL of bacterial lysis buffer (MagNA Pure Bacteria Lysis Buffer; Roche Diagnostics, Indianapolis, IN), 10 μL of 0.5 μg/μL lysostaphin (Sigma-Aldrich, St. Louis, MO), and 10 μL of 0.32 μg/μL lysozyme (Sigma-Aldrich). Specimens were vortex mixed and incubated in a heat block at 37°C for 30 minutes. Proteinase K at 20 mg/mL (20 μL; MagNA Pure LC Proteinase K; Roche Diagnostics) was added, and specimens were vortex mixed and incubated in a heat block at 65°C for 10 minutes. Specimens were vortex mixed and placed at −80°C for 10 minutes and then at 95°C for 5 minutes before a final nucleic acid extraction using the MagNA Pure LC−DNA Isolation Kit I (Roche Diagnostics), as per the manufacturer's instructions. Positive and negative control specimens were extracted along with the mock whole blood specimens for quality control purposes.

Nucleic acid was extracted from each of the 16 *S. enterica* serovar specimens using the MagNA Pure LC−DNA Isolation Kit I, according to the manufacturer's instructions. Positive and negative control specimens were extracted along with the *S. enterica* serovar specimens for quality control purposes.

Primer Design and Sequence Analysis

Primers used for 16S PCR and HRMA analysis were designed as described.[24–28] For HRMA analysis, typhoidal and nontyphoidal *S. enterica* species sequences for potential PCR products generated by the V1, V3, and V6 primers were analyzed using BioEdit (Ibis Biosciences, Carlsbad, CA) to determine whether sequence differences between different species of *S. enterica* were substantial enough for differentiation with HRMA. From this analysis, it was determined that using a single set of primers for the V1, V3, or V6 regions alone would be insufficient to differentiate the *S. enterica* species, and that primers for all three regions would be required to provide unique identification codes for typhoidal and nontyphoidal *S. enterica* species.

16S PCR and HRMA

Extracted whole blood specimens from each *S. enterica* serovar were tested for the presence of eubacterial DNA by 16S PCR, using universal primers and uniprobe, as previously described.[24,25] Positive and negative controls were included for quality control purposes and for negative cutoff determination. Both specimens and controls were analyzed in triplicate. Briefly, 16S PCR was performed with primers p891F (5′-TGGAGCATGTGGTTTAATTCGA-3′) and p1033R (5′-TGCGGGACTTAACCCAACA-3′) in a total volume of 50 μL, which comprised 30 μL of PCR master mix and 20 μL of sample input. PCR master mix contained 25 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 1.5 μL of 67 μmol/L forward primer and reverse primer. The 2× TaqMan universal PCR master mix and the primers underwent an ultrafiltration step, as previously described.[24,25] After ultrafiltration, 1 μL of 2.5 U of AmpliTaq Gold LD (Applied Biosystems) and 1 μL of 10 μmol/L probe were added to make up the final master mix before sample addition. PCR conditions were performed as previously described.[24,25]

For serovar differentiation, 16S PCR was used, as previously described,[24,25] to determine the presence of eubacterial DNA in each *S. enterica* serovar specimen. After 16S PCR, each *S. enterica* serovar specimen that tested positive for eubacterial DNA by 16S PCR was analyzed using HRMA, as previously described,[26–28] for serovar identification. *Salmonella enterica* serovar specimens, along with appropriate control specimens for each of the nine different serovar groups, were assayed in triplicate for each hypervariable region: V1, V3, and V6. Briefly, every HRMA analysis was performed in a 10 μL total volume composed of 8 μL of PCR master mix and 2 μL of target input. The PCR master mix contained 4 μL of

2.5× LightScanner Master Mix (Idaho Technology, Salt Lake City, UT) and 2 μL of LightScanner Reagent Grade water (Idaho Technology). A total of 1 μL of 1.5 μmol/L forward primer and reverse primer for V1, V3, and V6 regions[26–28] was added to each separate reaction. Each PCR analysis contained one primer pair. The PCR was performed using reaction conditions as previously described.[26–28]

Data Analysis for LOD and Serovar Differentiation

For LOD determination, 16S PCR−positive results were defined as reactions having a C_T value <32. This C_T cutoff was determined by the appropriate negative controls and an exponential increase in fluorescence above baseline. Positive amplification was confirmed through analysis of multicomponent data. A positive 16S PCR result indicated the presence of eubacterial DNA in the nucleic acid extracted from the specimen. The LOD, calculated as CFU/mL, for each *S. enterica* serovar was determined using the 16S PCR results and calculated concentrations from plate titers.

For serovar differentiation via HRMA, 16S PCR−positive results were defined as reactions having a C_T value <32. This C_T cutoff was determined as previously described. A positive 16S PCR result indicated the presence of eubacterial DNA in the nucleic acid extracted from the *S. enterica* serovar specimen. For HRMA, positive results were defined as specimens having melt curve profiles that matched control organism melt curve profiles.[26–28] Briefly, HRMA was performed on the LightScanner instrument (Idaho Technology) using a temperature gradient from 60°C to 95°C, with data acquisition performed for every 0.1°C increase in temperature. LightScanner Software version 2.0 (Idaho Technology), was used for data analysis, and the negative filter was first used to identify negative controls and any failed PCRs, followed by fluorescence normalization to minimize the variations in fluorescence magnitude between samples due to differences in starting template concentration. Derivative plots were generated to assess the number of melting peaks. Analysis subsets (V1, V3, and V6) were defined by the primer sets used for amplification. Difference plots were then generated using the autogrouping software feature to group all positive samples with a similar curve shape within the same analysis subset. A unique letter code was manually assigned for each group identified, starting with the letter *a* and progressing alphabetically. A combination of each letter from each of the variable regions was then accumulated to provide a signature code for each organism. Initial validation for *Salmonella* was performed previously[27] with one organism, and this study expanded this finding to increase the differentiation capability and specificity of HRMA for *S. enterica* species.

Results

The LOD for each individual *S. enterica* serovar mock whole blood specimen analyzed by 16S PCR is shown in Table 1. The overall LOD of 16S PCR for all nine typhoidal and nontyphoidal *S. enterica* serovars analyzed was <10 CFU/mL. The *S. enterica* serovars with the lowest LOD (<1 CFU/mL) are *S. enterica* serovar Paratyphi C, CVD P53; *S. enterica* serovar Choleraesuis variant Kunzendorf, P159; and *S. enterica* serovar Newport, 361. The *S. enterica* serovars with the highest LOD (9 CFU/mL) are *S. enterica* serovar Typhi, CVD A19; and *S. enterica* serovar Paratyphi A, EAR 6473.

All 16 typhoidal and nontyphoidal *S. enterica* serovars in the nine species groups tested positive for eubacterial DNA by 16S PCR. The *S. enterica* serovar specimens testing positive for eubacterial DNA were then analyzed by HRMA. Specimens from each of the nine groups of *S. enterica* serovars generated unique grouping codes when analyzed by HRMA (Table 2).

Discussion

In this study, the analytical sensitivities of a previously described broad-based 16S PCR[24,25] are reported for nine typhoidal and nontyphoidal *S. enterica* serovars (Table 1) in mock whole blood specimens. The overall analytical sensitivity reported, <10 CFU/mL for all serovars evaluated, is within the lower limits of clinically relevant concentrations of *S. enterica* infections. These analytical sensitivities, albeit generated by mock whole blood specimens, might have potential clinical utility in direct testing of blood samples from patients with suspected *S. enterica* infections. However, this practical utility needs to be evaluated by a clinical test trial.

In addition to evaluating the analytical sensitivities, the serovar differentiation capability of the 16S PCR coupled with HRMA[26–28] for 16 typhoidal and nontyphoidal *S. enterica* organisms, making up nine different serovar groups, was analyzed in this study. The grouping codes generated by the analysis of these serovars using 16S PCR coupled with HRMA (Table 2) demonstrated that each of the nine *S. enterica* serovar groups analyzed generated unique grouping codes. These unique grouping codes enable serovar-level differentiation between all nine of the *S. enterica* serovar groups. The data generated by HRMA were highly similar to what were observed by performing sequence analysis, and confirmed the need to use primers for V1, V3, and V6 regions of the 16S rRNA gene to generate unique grouping codes for all *S. enterica* serovars analyzed. For example, *S. enterica* Typhi, Paratyphi B, Typhimurium, and Newport all produced the same melting curve for the V1 region of the 16S rRNA gene, consistent with observations from sequence analysis of that region.

The level of identification achieved by the 16S PCR coupled with HRMA could be considered comparable to that of serotyping performed by public health laboratories. Other methods that could be used for species differentiation of *S. enterica* responsible for an infection would include multilocus sequence typing, matrix-assisted laser desorption/ionization time of flight (MALDI-TOF), or next-generation

sequencing. These methods of species-level identification have some advantages over using HRMA for species-level identification. MALDI-TOF has been successfully used for rapid discrimination of *S. enterica* serovar Typhi from nontyphoidal serovars; however, this was performed from positive blood culture specimens, adding delays because of the culture process, as opposed to detecting the organism directly from patient blood specimens.[23] Next-generation sequencing is certainly more informational than MALDI-TOF or HRMA for species-level identification, but the time and technical requirements for these methods can limit their applicability in clinical settings, or settings where treatment decisions are required in a timely manner. HRMA has a simpler testing format than many molecular methods, is substantially faster than blood culture and other techniques, is a closed-tube system, and has the capability to provide identification of pathogenic organisms directly from human clinical specimens.[29] Although HRMA has disadvantages, notably the inability to resolve specimens with polymicrobial infections or high levels of contaminating DNA sequences, it potentially offers diagnostic information that would allow for timely, accurate results that would improve diagnosis and treatment of serious *S. enterica* bloodstream infections.

The broad-based 16S PCR assay demonstrated excellent analytical sensitivity for detecting nine common serovars of typhoidal and nontyphoidal *S. enterica* from mock whole blood specimens, whereas the HRMA component of the assay illustrated accurate differentiation of nine *S. enterica* serovar groups. Detection of these emerging pathogens in febrile, clinically ill patients will be required in future studies to determine the true clinical utility of this molecular diagnostic method for the diagnosis and surveillance of *S. enterica* species infections.

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