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Research Note

A Rapid Screen of Broth Enrichments for *Salmonella enterica* Serovars Enteritidis, Hadar, Heidelberg, and Typhimurium by Using an Allelotyping Multiplex PCR That Targets O- and H-Antigen Alleles

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ABSTRACT

Salmonella continues to cause significant foodborne outbreaks, best illustrated with recent outbreaks associated with peanut butter, raw tomatoes, and serrano peppers. To ascertain the likely source of the outbreak, bacterial typing is essential to this process. While PCR has become an important detection tool for pathogens in foods, PCR can also identify strain differences by targeting gene(s) or sequences exhibiting polymorphisms or variability in its distribution within the bacterial population. Over 2,500 *Salmonella enterica* serovars identified based on antigenic differences in lipopolysaccharide and flagellin have been identified to date. We developed an allelotyping PCR scheme that identifies the O and H alleles associated with *S. enterica* serovars Enteritidis, Hadar, Heidelberg, Typhimurium, and others, with the same antigen alleles but in different O- and H-allele combinations (e.g., *S. enterica* Kentucky), and validated it as a screen to identify samples contaminated with these *Salmonella* serovars. We correctly identified poultry samples containing *S. enterica* serovars Enteritidis, Kentucky, and Typhimurium from our multiplex screen of primary enrichments of environmental drag swabs. PCR agreed well (kappa values = 0.81 to 1.0) with conventional serotyping methods used to type salmonellae isolated from primary enrichment. Coupled with *Salmonella*-specific PCR, such as *invA,* this allelotyping PCR could prove useful in the identification of *Salmonella* and specific *S. enterica* serovars present in foods or the environment and could decrease the time and cost associated with conventional serotyping methods.

Salmonella is one of the leading causes of human foodborne infection in the United States, responsible for 14.9 cases per 100,000 persons in the United States during 2006 *(28)*. In order to decrease human illness, measures need to be taken to reduce or eliminate the source(s) of infection. Identifying sources of *Salmonella* requires not just determining its presence, but also discerning the phenotypic or genotypic differences within the bacterial population to delineate a bacterial species to serovar or strain level.

Salmonella enterica serovar designation is based on the differences in the lipopolysaccharide O antigens, phase 1 (H1) and phase 2 (H2) flagellar antigens *(1),* and the specific O- and H-antigen combinations that constitute the 2,541 recognized *Salmonella* serovars. For example, *Salmonella* with O:H1:H2-antigen formula 4,5,12:i:1,2 is designated serovar Typhimurium, while serovar Heidelberg is given to an isolate with a similar antigenic formula, except the H1 antigen is r instead of i. Despite the diversity of *S. enterica* serovars, 10 *S. enterica* serovars—Typhimurium, Enteritidis, Newport, Heidelberg, Javiana, 4,(5),12:i:–, Montevideo, München, Saint Paul, and Braenderup—account for 66% of all cases of laboratory-confirmed salmonellosis in the United States *(1)*. In fact, *S. enterica* serovars Enteritidis and Typhimurium alone account for 79% of salmonellosis reported worldwide *(2)*. *S. enterica* serovar Enteritidis has been implicated in many foodborne outbreaks associated with the consumption of grade A table eggs *(27)*. Therefore, finding this *S. enterica* serovar in a commercial egg layer operation or primary chicken breeder flocks can adversely affect the sale, profitability, import, and export of product *(4),* so much so that quality assurance programs have been developed to monitor flocks for this *Salmonella* serovar, as well as other important pathogenic serovars in chicken broiler, breeder, and layer flocks *(3)*.

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To control and reduce salmonellosis in humans, it is important to generate rapid and reliable methods for detecting and typing *Salmonella,* in order to trace back the source of contamination. PCR has become an important tool for detecting the presence of microbial pathogens in foods *(23)* by identifying target gene(s) or nucleotide sequence(s) unique to a specific pathogen *(26),* serovar *(24),* or strain *(16)*. PCR can assist in making important decisions regarding presumptive identification of a pathogen *(20)* and the placement of efforts and resources toward isolation of the suspect pathogen from foods *(9)* or the farm environment *(22)*. As multiple *Salmonella* serovars can be present in the same farm, hatchery, and even the same animal *(6, 7, 13, 21),* it would be helpful to have a tool that can rapidly assess what, and how many, *Salmonella* serovars might be present in any given sample. Once isolated, determining the *Salmonella* serovar requires identifying specific O, H1, and H2 antigens among the repertoire of 46 lipopolysaccharide O antigens and 86 flagellar H antigens that compose the 2,541 reported *S. enterica* serovars. Therefore, serotyping can be a formidable task because of the numerous antisera required and the expertise necessary for interpreting weak tube agglutination reactions, thereby limiting its efficacy as a large-scale screening tool. As these antigenic differences are a reflection of the genetic divergence within the O- and H-antigen gene alleles, a targeted PCR could exploit these differences and identify specific *Salmonella* serovar prior to (or without the need to) isolate *Salmonella* from the sample. This article describes multiplex PCR as a screen to identify *S. enterica* serovars Enteritidis, Hadar, Heidelberg, Typhimurium, and related serovars present in the poultry environment.

MATERIALS AND METHODS

Isolation and serotyping of *Salmonella*. Two commercial broiler farms (two poultry houses per farm) were sampled by using drag swabs *(6)*. Five drag swabs were used to sample each poultry house for every farm visit ($n = 4$). Samples were used to inoculate tetrathionate–brilliant green enrichment broth (Difco, Becton Dickinson, Sparks, MD), and the enrichment broth was incubated at 41°C for 18 h *(5)*. After the enrichment step, 1 ml of tetrathionate–brilliant green broth was taken for each sample and processed for PCR, as described by Liu et al. *(22)*. A loopful of the enrichment broth was also streaked onto xylose–lysine–Tergitol 4 and brilliant green with novobiocin (Difco, Becton Dickinson), which was followed by overnight incubation at 37°C *(15)*. Suspect *Salmonella* colonies were confirmed by using poly(O) *Salmonella*-specific antiserum (Difco, Becton Dickinson). Serotyping was done by using standard serological typing procedures for *Salmonella* O, H1, and H2 antigens *(10)*.

Multiplex PCR for *Salmonella* O-, H1-, and H2-antigen genes and alleles. We identified and targeted allele variable regions within the *wba* O-antigen biosynthesis operon in the design of our specific PCR primer sets to identify *Salmonella* serogroups A/D1, B, C1, C2, and E1 *(18)*. PCR primer sets were also designed to identify the variable regions within the H1- and H2-antigen genes specific to H1 alleles i, g,m, r, and $z_{10,}$ and H2 alleles for antigen complexes 1,2; 1,5; 1,6; 1,7 and e,n,x; e,n,z_{15}. The H1-allelotyping PCR was designed as two multiplexes where H1–1 PCR identified alleles for H1 antigens i or g,m, and the H1–2 multiplex PCR detected alleles for H1 antigens r or z_{10}. The H2 multiplex PCR was designed to identify the two H2-antigen complexes 1,2; 1,5; 1,6; 1,7 and e,n,x; e,n,z_{15}. We have validated the specificity of each PCR primer set against a collection of *Salmonella* isolates ($n = 209$), which represents 43 serovars defined by six major O serogroups, 20 H1 antigens, and eight H2-antigen alleles *(18)*. Details of multiplex PCRs are described elsewhere *(18)*. Since the H2 multiplex PCR cannot distinguish monophasic from other possible H2 antigens (e.g., z_6) *(18),* a presumptive serovar designation was given based on information concerning *S. enterica* serovar prevalence for specific animal species *(25)*.

Statistics. The kappa statistic was calculated to evaluate the agreement between the classical serotyping systems and multiplex PCR. Sensitivity and specificity were also calculated for each contingency table. Stata, version 10.1 (StataCorp, L.P., College Station, TX), was used to perform statistical analysis.

RESULTS

Our multiplex PCR assay focuses on the detection of unique nucleotide sequences associated with certain *Salmonella* flagellar gene alleles or O-antigen gene clusters present in specific *S. enterica* serovars. The amplicon's size corresponds to a specific antigen gene or allele. For example, using the O multiplex PCR, the primers are designed to produce PCR amplicons of 624, 561, 341, 397, and 281 bp for *Salmonella* belonging to serogroups A/D1, B, C1, C2, and E1, respectively *(18)*. Likewise, H1–1 multiplex PCR amplifies PCR products of 309 and 508 bp for all salmonellae with H1 g,m or i alleles, respectively; H1–2 multiplex PCR produces 169- or 363-bp amplicons corresponding to H1 r and z_{10} alleles; and H2 1,2:e,n,x multiplex produces 294 or 152 bp for any *Salmonella* with either 1,2; 1,5; 1,6; 1,7 H2-antigen complex or e,n,x, e,n,z_{15} H2-antigen complex, respectively. Therefore, for each multiplex PCR, amplicon size corresponds to a specific antigen or allele, and the amplicons identified by combined multiplex PCRs correspond to specific O, H1, and H2 alleles that together identify the serovar *(18)*. Similarly, the identity of several other *Salmonella* serovars can be ascertained based on 1:1 correspondence of amplicon's size with the O and/or H allele(s) targeted by PCR. While the multiplex PCRs were designed to specifically detect *S. enterica* serovars Enteritidis, Hadar, Heidelberg, and Typhimurium *(18),* they can also detect other *S. enterica* serovars bearing the same O, H1, and H2 alleles, but in different combinations. Therefore, as a screen, this allelotyping PCR can alert us to single, and possibly multiple, *Salmonella* serovars based on detection of the individual O, H1, and H2 alleles.

We applied our multiplex PCR as a screen of primary enrichment broths for the major *S. enterica* serovars Enteritidis, Hadar, Heidelberg, and Typhimurium, as well as other *S. enterica* serovars that possess the same O-, H1-, and H2-antigen gene alleles. Primary enrichments were done with tetrathionate–brilliant green broth for 80 drag swabs collected from four commercial poultry houses sampled for *Salmonella*. Of the 80 primary enrichments, 40 were culture positive for *Salmonella*. Of the 40

TABLE 1. *Comparison of allelotyping PCR versus serotyping for the identification of specific* Salmonella enterica *serovars in tetrathionate–brilliant green enrichment*

Salmonella enterica serotype	Multiplex PCR	Serotyping[a]	Statistics		
			Kappa[b]	Sensitivity[c]	Specificity[d]
Typhimurium	27/80[e,f]	32/80	0.81	0.81	0.98
Enteritidis	3/80[g]	3/80	1.00	1.00	1.00
Kentucky	4/80[h]	5/80	0.88	0.80	1.00

[a] *Salmonella* isolated from primary enrichment was typed by using standard serotyping methods.
[b] The agreement between multiplex PCR screens of primary enrichment broths and conventional serotyping of *Salmonella* isolated from the primary enrichment was evaluated by calculating the kappa statistic.
[c] Probability of a positive result by PCR among isolates that are positive by serotyping.
[d] Probability of a negative result by PCR among isolates that are negative by serotyping.
[e] Overnight tetrathionate–brilliant green enrichment broths positive for *Salmonella* O-antigen B, H1-antigen i, and H2-antigen complex 1,2; 1,5; 1,6; 1,7 by multiplex PCR.
[f] One of 27 PCR positive samples was culture negative for *Salmonella*.
[g] Overnight tetrathionate–brilliant green enrichment broths positive for *Salmonella* O-antigen D1 and H1-antigen gm by multiplex PCR. No PCR amplicons generated with *fljB* multiplex PCR for H2-antigen complexes 1,2; 1,5; 1,6; 1,7 or e,n,x; e,n,z_{15}.
[h] Overnight tetrathionate–brilliant green enrichment broths positive for *Salmonella* O-antigen C2 and H1-antigen i by multiplex PCR. No PCR amplicons generated with *fljB* multiplex PCR for H2-antigen complexes 1,2; 1,5; 1,6; 1,7 or e,n,x; e,n,z_{15}.

Salmonella isolated from primary enrichments, 32 were *S. enterica* serovar Typhimurium, 3 were serovar Enteritidis, and 5 were serovar Kentucky, based on conventional serotyping. Multiplex PCRs identified *S. enterica* O and H alleles associated with serovars Enteritidis, Kentucky, and Typhimurium directly from 34 of the same 40 primary enrichment broths that were culture positive for *Salmonella*. One (2.5%) false positive and seven (18%) false negatives were reported for the multiplex PCR. PCR agreed well (kappa values = 0.81 to 1.0) with conventional serotyping methods used to type salmonellae isolated from primary enrichment (Table 1).

DISCUSSION

PCR has become a very useful tool in detecting or typing enteric pathogens, such as *Campylobacter, Escherichia coli* O157, *Salmonella,* and *Vibrio cholerae (11, 12, 14, 17–19, 24)*. However, traditional, microbiological approaches to isolation and identification of foodborne pathogens often deal with complex samples and larger volumes that PCR cannot accommodate due to problems with PCR inhibitors or detection limits *(23)*. An initial enrichment step can overcome these limitations by increasing cell density to levels well above the PCR's threshold for detection to accommodate both the dilution of PCR inhibitors and detection of the pathogen *(22, 24)*. As enrichments are a common component of many food and diagnostic microbiology protocols, we envisioned the addition of our multiplex PCR as a screen after enrichment to assist in a presumptive identification and decision on samples toward which to focus efforts in isolating *Salmonella* serovar(s) present in the sample.

We were able to apply the allelotyping PCR to identification of *S. enterica* serovars Enteritidis, Kentucky, and Typhimurium, in screens of primary enrichments. There was excellent agreement between our allelotyping multiplex PCRs of overnight enrichment broth and serotyping of salmonellae isolated from primary enrichment in tetrathionate broth (kappa values = 0.81 to 1.00). Depending on the *S. enterica* serovar, sensitivity ranged between 0.80 and 1.00, and between 0.98 and 1.00 for the test's specificity. Reporting false positives (PCR positive, culture negative) with our multiplex PCR was expected, since PCR cannot distinguish between live, injured, or dead *Salmonella* cells. In fact, we have used *Salmonella invA* PCR screen of tetrathionate–brilliant green enrichments to improve our isolation of *Salmonella* from environmental drag swabs by focusing our delayed secondary enrichments on PCR-positive, culture-negative samples, increasing our isolations by 20% *(22)*. Another possible explanation for the false positive is nonspecific amplicons, but these amplicons would have to be the same size expected for each primer set used to detect the O and H alleles, and these nonspecific amplicons would need to migrate to the same gel position, as did the positive controls, which were included with every PCR. False negatives were not attributed to genetic differences in the *S. enterica*–targeted O and H alleles, since multiplex PCR was able to identify correctly the *S. enterica* serovar for all isolates obtained from the primary enrichments, including those enrichments that were PCR negative for the O and H alleles screened. It is more likely that carryover calcium carbonate from the tetrathionate interfered with PCR, or the number of salmonellae present after the initial enrichment was below the level of detection *(17, 22)*.

There are limitations with the multiplex primers used in this study. First, several primers do not have the specificity needed to differentiate related alleles (e.g., serogroup A versus D1, or e,n,x versus e,n,z_{15}) *(18)*. Although the *S. enterica* serovar in question may be rare (e.g., *S. enterica* serovars Hadar [H2: e,n,x] versus Glostrup [H2: e,n,z_{15}]), a reporting laboratory may require confirmatory testing *(18)*. Second, negative PCR results with H2 allelotyping primers may be attributable to absence of the H2 flagellin gene *fljB* in monophasic salmonellae, or have other H2 alleles.

Addition of a second PCR that detects *fljB* in all biphasic *S. enterica* serovars would be able to determine the former possibility *(8),* while other H2 allelotyping primers could identify the other H2 alleles *(11, 12)* and expand the potential *S. enterica* serovars identified by PCR.

Diagnostic laboratories can rapidly determine if sample(s), after enrichment, contain one of four major *S. enterica* serovars (Enteritidis, Hadar, Heidelberg, and Typhimurium) by using this allelotyping PCR screen in 4 to 6 h versus the minimum 72 h needed to isolate and identify the *Salmonella* serovar by using standard microbiological methods. As our PCRs are designed to detect multiple O, H1, and H2 alleles, they can potentially alert the laboratory to the presence of multiple *Salmonella* serovars in the same sample. Although the allelotyping PCR may not be able to determine definitively which are present in this scenario, this information can at least assist in the decisions as to whether to pick one or more presumptive *Salmonella*-positive colonies for additional serotyping.

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