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One-Step Identification of Five Prominent Chicken *Salmonella* Serovars and Biotypes

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Based on bacterial genomic data, we developed a one-step multiplex PCR assay to identify *Salmonella* and simultaneously differentiate the two invasive avian-adapted *S. enterica* serovar Gallinarum biotypes Gallinarum and Pullorum, and the most frequent, specific, and asymptomatic colonizers of chickens, serovars Enteritidis, Heidelberg, and Kentucky.

Strains of most *Salmonella* serovars are zoonotic. Approximately 90% of human salmonellosis is caused by ingestion of contaminated food products of animal or plant origin (1). With >19,000 reported cases in the United States in 2013, *Salmonella* remains the most frequently isolated bacterial food pathogen, as determined by the surveillance network FoodNet, which pools the data of 10 U.S. monitoring sites (2). In parallel with the rise in poultry consumption over the years in the United States, the commercial poultry industry has grown impressively, reaching >9 billion raised and processed broiler chickens and production of >77 billion table eggs per year, as indicated for 2009 (3). *Salmonella* is a frequent asymptomatic intestinal colonizer of poultry. Stress or underlying diseases in young birds create optimal conditions for productive horizontal transmission of *Salmonella* sp. Data from the USDA-FSIS suggest that every fourth raw chicken part is likely contaminated with *Salmonella* (2). Moreover, major *Salmonella* serovars can spread to reproductive organs, leading to vertical transfer of the bacteria and egg-related salmonellosis (4, 5). Accordingly, consumption of poultry and egg represents a significant source of *Salmonella* infections in the United States.

Four *Salmonella* serovars are of particular concern to the poultry industry, namely, Enteritidis, Heidelberg, Kentucky, and Gallinarum (6). *S.* Gallinarum is an invasive agent of chicken salmonellosis that results in high mortality and morbidity, with biotype Pullorum (*S.* Pullorum), which causes white diarrhea in young chickens (pullorum disease), and biotype Gallinarum, which is responsible for fowl typhoid (7). Although this serovar remains endemic in many countries, it has essentially been eradicated through culling programs in the domestic fowl industry of the United States and several other developed countries. *S.* Gallinarum can colonize and/or cause disease in various domestic and wild birds, which might explain its occasional detection in backyard birds of developed countries (8). In recent years, *S.* Enteritidis became the most frequently isolated serovar in poultry and from food-borne outbreaks linked to poultry products in developed countries (9). This serovar was suggested to have filled the ecological niche vacated by the eradicated *S.* Gallinarum biotypes Pullorum and Gallinarum (10). Lately, *S.* Heidelberg has become another major serovar responsible for food-borne infections from poultry products (11, 12) and one of the most common serovars obtained from nonclinical chicken isolates (9, 13, 14). *S.* Kentucky is the most common serotype isolated from chickens and the second most common serotype found among retail chicken products in the United States. It has rarely been reported in human cases in North America (15, 16), although this may change with the worldwide spread of the ciprofloxacin-resistant ST198 (17).

Here, we describe a simple one-step multiplex PCR method to identify major chicken *S. enterica* subsp. *enterica* serovars. The approach was based on designing primers that specifically amplify unique sets of *Salmonella* spp. and serovar-associated DNA sequences in one PCR tube (Table 1), taking advantage of 3,161 available *Salmonella* genomes, including strains from the serovars Enteritidis (369 genomes), Heidelberg (154), Kentucky (63), and Gallinarum (8 biotype Pullorum and 4 biotype Gallinarum) and of 2,563 genomes from 104 other serovars (http://www.ncbi.nlm.nih.gov/genome/genomes/152?). The desired specificities were checked by using BLAST (NCBI, nonredundant nucleotide collection). Strains of the *Salmonella* genus and Gallinarum biotypes were identified by primers for differently conserved DNA segments in their *bcf* and *ste* fimbrial usher genes, respectively (20). Specific primers for serovar Gallinarum biotype Gallinarum were made by taking advantage of a deletion of 4 nucleotides in *steB* of biotype Pullorum. Other specific DNA signatures served as primer targets to separate serovars Enteritidis, Heidelberg, and Kentucky. Briefly, for the multiplex PCR, pure template DNA (1 to 5 ng per reaction; MagNA Pure LC DNA isolation kit III; Roche Life Sci., India-

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TABLE 1 List of primers and concentrations used for PCR, with targeted DNA and amplicon sizes

Primer	Sequence (5′ to 3′)	Final primer concn (pmol/ml)	Targeted gene or locus[a]	Targeted DNA (species, serovar)	Amplicon size (bp)	Accession no. and nucleotide segment	Reference
bcfC-F	GGGTGGGCGGAAAACTATTTC	0.6	*bcfC*	*S. enterica*	993	AM933172	This study
bcfC-R	CGGCACGGCGGAATAGAGCAC					25665–26657	
heli-F	ACAGCCCGCTGTTTAATGGTG	2	ORF (predicted helicase)	Heidelberg	782	CP005995	This study
heli-R	CGCGTAATCGAGTAGTTGCC					3226024–3226805	
steB-F	TGTCGACTGGGACCCGCCCGCCCGC	2	*steB*	Gallinarum biotype Gallinarum[b]	636	AM933173	18
steB-R	CCATCTTGTAGCGCACCAT					2976016–2976651	
rhs-F	TCGTTTACGGCATTACACAAGTA	2.6	rhs locus	Gallinarum	402	AM933173	This study
rhs-R	CAAACCCAGAGCCAATCTTATCT					334109-334510	
sdf-F	TGTGTTTTATCTGATGCAAGAG	2.6	sdf locus	Enteritidis	293	AF370716	19
sdf-R	CGTTCTTCTGGTACTTCAGATGAC					4950–5242	
gly-F	TTCCAATTGAAACGAGTGCGG	2.6	ORF (putative membrane protein)	Kentucky	170	ABEI01000007	This study
gly-R	ACTAACCGCTTGGGTTGTTGCTGT					116981–117150	

[a] ORF, open reading frame.
[b] Absent in biotype Pullorum (accession no. CP006575, locus_tag = I137_00945) but also present in Enteritidis, Heidelberg, Kentucky, and group 1 serovars, as listed in Table 2.

napolis, IN) or crude DNA (~75 ng per reaction, from bacterial suspensions boiled for 5 min, 10^7 CFU/μl dH_2O, using 1 μl of supernatant after centrifugation) was amplified with *Taq* DNA polymerase and a final concentration of 1.5 mM Mg^{2+} (Choice-*Taq* Blue; Denville Sci., Inc., South Plainfield, NJ) using standard protocols. The PCR (25 cycles with an annealing temperature of 56°C) was performed with a Hybaid thermal cycler (Thermo Fisher Sci., Waltham, MA). The specificity and compatibility of the primer sets in a multiplex PCR were assessed using genomic DNA from 128 *Salmonella* strains that included a total of 34 different serovars, as well as from 3 *Escherichia coli* and 2 *Yersinia* spp. as negative-control strains (see Table S1 in the supplemental material).

Agarose gel electrophoresis profiles for the different amplicon sets are shown with representative strains in Fig. 1, and the results for all the strains are listed in Table 2. All of the *Salmonella* strains were recognized as such, as were strains of the Gallinarum biotypes and the Enteritidis, Heidelberg, and Kentucky serovars. Thus, the obtained experimental results were in agreement with the genomic information used for the primer designs and validated the proposed identification of *S. enterica* and the serovar/biotype differentiation among major chicken isolates.

Routine screening of flocks for the presence of *Salmonella* can be done by conventional serology, which is expensive, time consuming, and labor intensive. Based on the restricted number of major serovars found in chickens, extensive molecular techniques are not always cost-effective, and simpler, focused approaches may serve as rapid early diagnostic tests. Here, we took advantage of a small gap in the gene *steB* of biotype Pullorum that was predicted by genomic analysis (20) to design primers that hybridize to biotype Gallinarum but not Pullorum DNA, permitting a one-step PCR differentiation of the two biotypes (Table 2). This method shortened a previously described two-step technique (21). The addition of primers for additional chicken-associated serovars all in one multiplex PCR analysis is useful for the diagnosis of *Salmonella* in these birds. Although the designed probes are specific for the identification of serovars Heidelberg, Enteritidis, and Gallinarum, serovar Kentucky shares its PCR profile with serovar Albany, which is not a major chicken isolate in the United States (13, 14). If needed, the latter two serovars can be differentiated by a flagellin-specific PCR (see Fig. S1 in the supplemental material). Finally, rarer serovars for which genomic data are currently unavailable might share one of the described PCR profiles, but as such serovars are significantly less frequent in chicken isolates (13, 14), this would be of minor concern.

Taken together, this study used (i) genomic sequence data for *Salmonella* to design a chicken-specific multiplex PCR diag-

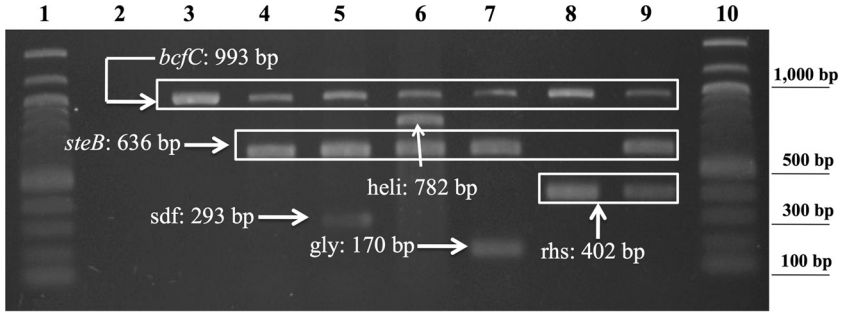


FIG 1 Agarose gel (1.5%) of multiplex PCR amplicons from different bacterial strains. Representative gel from three comparable experiments. Lanes 1 and 10, 100-bp DNA ladder (NEB, Ipswich, MA); lane 2, *Escherichia coli* (DH5a, negative control); lane 3, S. *enterica* group 2, according to Table 2; lane 4, *S. enterica* group 1, according to Table 2; lane 5, *S. enterica* serovar Enteritidis; lane 6, *S. enterica* serovar Heidelberg; lane 7, *S. enterica* serovar Kentucky; lane 8, *S. enterica* serovar Gallinarum biotype Pullorum; lane 9, *S. enterica* serovar Gallinarum biotype Gallinarum.

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TABLE 2 Bacterial strains used to confirm the specificity of the multiplex PCR assay

Salmonella enterica serovar and biotype[a]	Multiplex PCR positive for:					
	bcfC	heli	*steB*	rhs	sdf	gly
Heidelberg (2)	+	+	+	−	−	−
Enteritidis (11)	+	−	+	−	+	−
Kentucky (4)	+	−	+	−	−	+
Gallinarum biotype Gallinarum (16)	+	−	+	+	−	−
Gallinarum biotype Pullorum (7)	+	−	−	+	−	−
Others						
Group 1(68)[b]	+	−	+	−	−	−
Group 2 (20)[c]	+	−	−	−	−	−
Non-*Salmonella* strains (5)[d]	−	−	−	−	−	−

[a] Number of strains given in parentheses (see Table S1 in the supplemental material).
[b] Other *S. enterica* serovars (group 1) that have the same PCR profile: Paratyphi A (4 isolates), Paratyphi B var. Java (1), Agona (4), Abortusequi (2), Abortusovis (2), Saintpaul (3), Stanleyville (1), Typhisuis (2), Braenderup (5), Choleraesuis (24), Ohio (1), Thompson (1), Hadar (2), Muenchen (2), Newport (6), Berta (2), Dublin (2), Panama (1), Typhi (1), Agoueve (1), and Cerro (1).
[c] Other *S. enterica* serovars (group 2) that have the same PCR profile: Schwarzengrund (3 isolates), Typhimurium (2), Bareilly (1), Hartford (1), Montevideo (2), Oranienburg (3), Javiana (6), Mississippi (1), and Pomona (1).
[d] Three *E. coli* and two *Yersinia* strains.

nostic test and (ii) an extensive library of *Salmonella* strains and serovars to validate the specificity of the method for the identification and differentiation of major avian-associated serovars. This simple and economical test should be useful for specific screening of poultry flocks, particularly in developing countries, and of backyard flocks and game birds in developed countries.

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