One-step identification of five prominent chicken *Salmonella* serovars and biotypes

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Abstract:

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, as well as the most frequent, specific and asymptomatic colonizers of chickens, serovars Enteritidis, Heidelberg and Kentucky.

Keywords:

Chicken, *Salmonella*, *S.* Enteritidis, *S.* Heidelberg, *S.* Kentucky, *S.* Gallinarum, *S.* Pullorum, multiplex PCR

Strains of most *Salmonella* serovars are zoonotic. Approximately 90% of human salmonellosis results from ingestion of contaminated food products of animal or plant origin (1). With over 19,000 reported cases in the US for 2013, *Salmonella* remains the most frequently isolated bacterial food pathogen, as determined by the surveillance network FoodNet which pools the data of 10 US monitoring sites (2). In parallel to the rise of poultry consumption over the years in the US, the commercial poultry industry has grown impressively, reaching over 9 billion raised and processed broilers per year and a yearly production of over 77 billion table eggs, 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 suggests 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, poultry and egg consumption represent a significant source of *Salmonella* infections in the US.

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 resulting in high mortality and morbidity, with biotype Pullorum (*S.* Pullorum) which causes "white diarrhea" in young chicken (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 USA 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 a most frequently isolated serovar in poultry and from foodborne 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 foodborne infections from poultry products (11, 12), as well as one of the most common serovars obtained from non-clinical chicken isolates (9, 13, 14). *S.* Kentucky is the most common serotype isolated from chickens and the second most common one found among retail chicken product in the USA. It has been rarely reported in human cases in North America (15, 16), although this could change with worldwide spreading of the ciprofloxacin-resistant ST198 (17).

Here we describe a simple one-step multiplex polymerase chain reaction (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 serovar Enteritidis (369 genomes), Heidelberg (154), Kentucky (63), Gallinarum (8 biotype Pullorum and 4 biotype Gallinarum), and 2,563 genomes from 104 other serovars.

The desired specificities were checked by using BLAST (NCBI, non-redundant nucleotide collection). Strains of the *Salmonella* genus and Gallinarum biotypes were identified by primers for differently conserved DNA segments in the their *bcf* and *ste* fimbrial usher genes, respectively (18). 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 (1-5 ng per reaction; MagNA Pure LC DNA Isolation Kit III, Roche Life Sci., Indianapolis, IN) or crude DNA (approximately 75 ng per reaction, from bacterial suspensions boiled for 5 min, 10^7 CFU/µl dH_2O, using 1 µl supernatants 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 was assessed using genomic DNA from 128 *Salmonella* strains that included a total of 34 different serovars as well as three *Escherichia coli* and two *Yersinia* spp. as negative control strains (Suppl. Table 1).

Agarose gel electrophoresis profiles for each different amplicon sets are visualized with representative strains in Fig. 1 and the results for all the strains are listed in Table 2. All 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 primers' design 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, as well as time- and labor-consuming. Based on the restricted number of major serovars found in chicken, extensive molecular techniques are not always cost-effective, and simpler more focused approaches could serve as rapid early diagnostic tests. Here, we took advantage of a small gap in gene *steB* of biotype Pullorum that was predicted by genomic analysis (18) 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 (19). 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 USA (13, 14). If needed, these two serovars could be differentiated by a flagellin-specific PCR (Suppl. Fig. 1). Finally, rarer serovars for which genomic data are currently unavailable might theoretically share one of the described PCR profiles, but as such serovars are significantly less frequent in chicken (13, 14), this would be

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of minor concern.

Taken together, this study used (a) genomic sequence data for *Salmonella* to design a chicken-specific multiplex PCR diagnostic test and (b) 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 for developing countries or backyard flocks and game birds in developed countries.

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Table 1. List of primers and concentration used for PCR, with targeted DNA and amplicons sizes.

Primers	Sequences (5'to 3')	Final primer concentrations (pmole/ml)	Targeted genes or loci	Targeted DNA (species, serovars)	Amplicon sizes (bp)	Accession # and Nt segments
bcfC-F	GGGTGGGCGGAAAACTATTTC	0.6	*bcfC*	*S. enterica*	993	AM933172 25665- 26657
bcfC-R	CGGCACGGCGGAATAGAGCAC					
heli-F	ACAGCCCGCTGTTTAATGGTG	2	orf (predicted helicase)	Heidelberg	782	CP005995 3226024-3226805
heli -R	CGCGTAATCGAGTAGTTGCC					
steB -F	TGTCGACTGGGACCCGCCCGCCCGC	2	*steB*	Gallinarum biotype Gallinarum[1]	636	AM933173 2976016-2976651
steB-R	CCATCTTGTAGCGCACCAT					
rhs-F	TCGTTTACGGCATTACACAAGTA	2.6	rhs locus	Gallinarum	402	AM933173 334109-334510
rhs -R	CAAACCCAGAGCCAATCTTATCT					
sdf-F	TGTGTTTTATCTGATGCAAGAG	2.6	sdf locus	Enteritidis	293	AF370716 4950-5242
sdf-R	CGTTCTTCTGGTACTTCAGATGAC					
gly-F	TTCCAATTGAAACGAGTGCGG	2.6	orf "gly" (hypothetical protein)	Kentucky	170	ABEI01000007 116981 - 117150
gly-R	ACTAACCGCTTGGGTTGTTGCTGT					

[1] Absent in biotype Pullorum (Accession number CP006575, locus_tag="I137_00945"), but also present in serovars Enteritidis, Heidelberg, Kentucky and group 1 serovars, as listed in Table 2.

Table 2: Bacterial strains used for confirm the specificity of the multiplex PCR assay.

Salmonella enterica serovars and biotypes [1]	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) [2]	+	-	+	-	-	-
Others: Group 2 (20) [3]	+	-	-	-	-	-
Non *Salmonella* strains (5)[4]	-	-	-	-	-	-

[1] Numbers of strains in brackets (see Suppl. Table 1)

[2] 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), Cerro (1).

[3] Other *S. enterica* serovars (group 2) that have the same PCR profile: Schwarzengrund (3). Typhimurium (2), Bareilly (1), Hartford (1), Montevideo (2), Oranienburg (3), Javiana (6), Mississippi (1), Pomona (1).

[4] Three *E. coli* and two *Yersinia* strains.

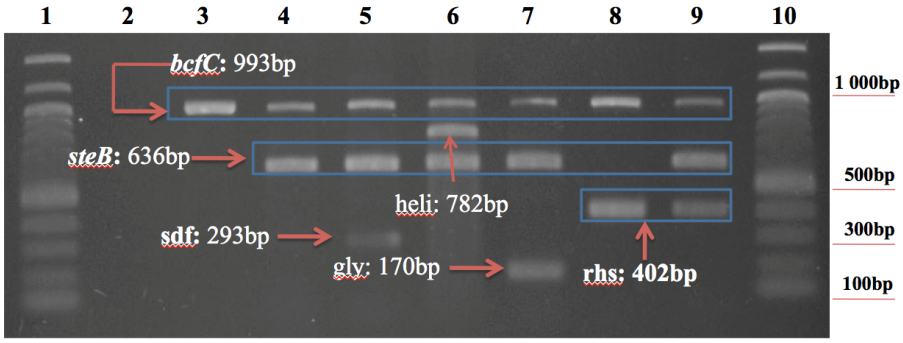

Fig. 1. Agarose gel (1.5%) of multiplex PCR amplicons from different bacterial strains. Representative gel from three comparable experiments. Lanes 1 and 10, 100 bp DNA ladder (NEB, Ipswich, MA); Lane 2, *Escherichia coli* (DH5a, negative control); Lane 3, S. *enterica* group 2, according to Table 2;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