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Original Articles

Subtyping of *Salmonella enterica* Subspecies I Using Single-Nucleotide Polymorphisms in Adenylate Cyclase

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Abstract

Methods to rapidly identify serotypes of *Salmonella enterica* subspecies I are of vital importance for protecting the safety of food. To supplement the serotyping method *dkgB*-linked intergenic sequence ribotyping (ISR), single-nucleotide polymorphisms were characterized within adenylate cyclase (*cyaA*). The National Center for Biotechnology Information (NCBI) database had 378 *cyaA* sequences from *S. enterica* subspecies I, which included 42 unique DNA sequences and 19 different amino acid sequences. Five representative isolates, namely serotypes Typhimurium, Kentucky, Enteritidis phage type PT4, and two variants of Enteritidis phage type PT13a, were differentiated within a microsphere-based fluidics system in *cyaA* by allele-specific primer extension. Validation against 25 poultry-related environmental *Salmonella* isolates representing 11 serotypes yielded a ~89% success rate at identifying the serotype of the isolate, and a different region could be targeted to achieve 100%. When coupled with ISR, all serotypes were differentiated. Phage lineages of serotype Enteritidis 13a and 4 were identified, and a biofilm-forming strain of PT13a was differentiated from a smooth phenotype within phage type. Comparative ranking of mutation indices to genes such as the tRNA transferases, the diguanylate cyclases, and genes used for multilocus sequence typing indicated that *cyaA* is an appropriate gene for assessing epidemiological trends of *Salmonella* because of its relative stability in nucleotide composition.

Highlights

- Allele-specific primer extension (ASPE) was validated as a subtyping method for *Salmonella enterica* by correctly identifying single-nucleotide polymorphisms (SNPs) in the *cyaA* gene from 25 strains isolated from the environment of poultry.
- More than 80 SNPs in the *cyaA* gene of *Salmonella* were tabulated, which indicates that *cyaA* is useful as a gene target for ASPE intended to provide some subtype information after the serotype is assigned by intergenic sequence ribotyping.
- Genes such as *cyaA* that have a nonsynonymous to total (NS/T) mutation index of less than 0.5 may be more conservative for establishing subtype within serotype than genes with a higher NS/T index.
- Genes with higher NS/T indices, such as the diguanylate cyclases, might be more sensitive for detecting rapidly emerging strain heterogeneity.

Introduction

A 42-year history of information on *Salmonella enterica* serotypes indicates it is essential to rapidly determine serotype and subtype of isolates from contaminated products to improve the safety of food (Liu *et al.*, 2011; Fabre *et al.*, 2012; CDC-NCEZID, 2013; Lettini *et al.*, 2014). Recently, a sequence-based method called *dkgB*-linked intergenic sequence ribotyping (ISR) became available to producers who want to independently screen for serotypes of *S. enterica* present on-farm, in processing facilities, and to make decisions about vaccination strategies (www.neogen.com/FoodSafety/NS_Sal.asp) (Guard *et al.*, 2012). However, methods that provide additional information about subtype in addition to serotype have not yet been developed to specifically complement ISR. Therefore, the goal of this research

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was to design and optimize a single-nucleotide polymorphism (SNP)-based hybridization assay using an increasingly available approach, namely allele-specific primer extension (ASPE) (Dunbar and Jacobson, 2007; Dunbar *et al.*, 2015). ASPE is conducted within a multiplexed bead-based capillary system requiring custom instrumentation (xMAP; Luminex, Austin, TX). A method that supports ISR by providing subtype should provide poultry food safety researchers and the industry with a rapid cost-effective pipeline that provides phage type and other information about genomic lineages occurring within serotype. By coupling ISR with a more sophisticated DNA-based method with a potential for multiplexing, such as ASPE, cost efficiency of processing hundreds to thousands of samples within a year should be facilitated, in part, because redundant processing of highly prevalent serotypes can be minimized.

Other genome-based methods have been developed to serotype the salmonellae in addition to ISR, and these include pulsed-field gel electrophoresis (Swaminathan *et al.*, 2001; Ji *et al.*, 2006), amplified fragment length polymorphism (Liebana *et al.*, 2002), multilocus sequence typing (MLST) (Sukhnanand *et al.*, 2005; Ji *et al.*, 2006; Liu *et al.*, 2011), multiple-locus variable-number tandem repeats (Lindstedt *et al.*, 2004), CRISPR analysis (Fabre *et al.*, 2012), and repetitive extragenic palindromic polymerase chain reaction (PCR) (Versalovic and Lupski, 2002). However, most of these methods do not correlate specific biological properties with SNPs that emerge between closely related strains within serotypes. For *S. enterica*, examples of using SNPs to pathotype *S. enterica* include (1) association of 16 SNPs with transition from an invasive phenotype to one that is environmentally prevalent, but epidemiologically inconsequential (Guard *et al.*, 2011), (2) impact on the predicted epitopes of flagella (McQuiston *et al.*, 2011), and (3) impact on antibiotic resistance (Song *et al.*, 2010). The importance of SNPs is emphasized by a fundamental concept of microevolution; specifically, a single base pair change can have as much impact on the ability of a pathogen to cause disease as does an exchange of chromosomal DNA of thousands of base pairs. An example of such microevolution is in *Listeria monocytogenes*, where SNPs were used to identify outbreak strains and to determine their incidence in ready-to-eat foods relative to environmental strains (Ducey *et al.*, 2007; Ward *et al.*, 2008; Van Stelten *et al.*, 2010).

One physiologically important gene that is suggested as a target for microevolutionary studies in *S. enterica* serotypes is adenylate cyclase (*cyaA*) ((Zhang *et al.*, 1996; Morales *et al.*, 2007). It has been associated with evolutionary trends in avian-adapted serotypes, such as *Salmonella* serotypes Pullorum and Gallinarum (Morales *et al.*, 2007). Several other factors make *cyaA* an attractive gene target for subtyping. These factors are as follows: (1) *cyaA* is central to energy production and metabolism (Lory *et al.*, 2004), (2) required for virulence (Curtiss III and Hassan, 1996), (3) it is present as one copy, and (4) it is associated with physiological change when mutated (i.e., reduced lethality, growth, and environmental persistence) (Kennedy *et al.*, 1996; Zhang *et al.*, 1996). In addition, the mutations within *cyaA* gene have potentially evolved as a coping mechanism during changing environmental conditions, allowing *S. enterica* serotypes to maintain critical functions required for baseline survival and infection potential (Aravind and Koonin, 1999; Wolfgang *et al.*, 2003; Baker and Kelly, 2004). To further understand if there is any impact by selecting *cyaA* over other genes for developing SNP analysis supporting ISR serotyping, BLAST analyses were used to collect pertinent information for characterization of three other sets of genes. Set 1 included 20 tRNA transferases (Ogle and Ramakrishnan, 2005), set 2 included 13 diguanylate cyclases (DGC) (Jenal and Malone, 2006), and set 3 included 23 genes commonly included in other genome studies. Results suggest that *cyaA* has characteristics favorable for supporting ISR and for being a reference gene for assessing heterogeneity occurring between strains associated with outbreaks.

Materials and Methods

BLAST for recovery of available S. enterica *subspecies I sequences*

A strategy for BLAST analysis was followed to find only unique *cyaA* sequences and then to translate those sequences into unique amino acid sequences. The reference genome for all BLAST searches was *Salmonella enterica* serovar Typhimurium LT2 (NC_003197.1). Specifically, gene STM3939 was used in BLAST searches of complete and draft genomes to obtain all *cyaA* sequences of record within *S. enterica* subspecies I between August 15 and September 20, 2015. The National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) was the database searched. The 175 entries for serovar Enteritidis from NCBI separated into three *cyaA* sequences, and other serovars also had multiple *cyaA* sequences. One other *cyaA* sequence for serotype Enteritidis is available, but it was not used in BLAST search calculations although it was included for development of the assay.

Gene *cyaA* is highly conserved within the genome of *S. enterica*, and there is only one copy (McClelland *et al.*, 2001). To determine how the nonsynonymous to total (NS/T) index of other genes compared to *cyaA*, three sets of genes were similarly analyzed (Fig. 1), and details for each gene are listed in Table 1. The tRNA transferases (Ogle and Ramakrishnan, 2005) and DGCs (Jenal and Malone, 2006; Romling, 2015) were selected because they are replicated in the genome of *S. enterica* several times (McClelland *et al.*, 2001). For the third set, genes were used that had been incorporated into various methods such as MLST (Fakhr *et al.*, 2005; Alcaine *et al.*, 2006; Tankouo-Sandjong *et al.*, 2007; Han *et al.*, 2010; Bell *et al.*, 2011; Stepan *et al.*, 2011; Seong *et al.*, 2012; Fresno *et al.*, 2014), as housekeeping genes in transcription assays (Csonka *et al.*, 1994; Galitski and Roth, 1997; Hensel *et al.*, 1999; Tedin and Norel, 2001; Olson *et al.*, 2007; Gilberthorpe and Poole, 2008; Malcova *et al.*, 2009; Chan *et al.*, 2012), or that were found to be part of a set of naturally mutated genes in *S. enterica* (Guard *et al.*, 2011). Geneious 8.1.6 software was used to conduct BLAST searches, multiple alignments, and translations. After conducting BLAST searches, the parameters for keeping a hit for further analysis included being within *S. enterica* subspecies I, showing no indication of truncation, and having an appropriate gene length.

S. enterica *strains used for ASPE and microsphere-based fluidics analysis of SNPs*

The five reference *S. enterica* strains used for initial development were Enteritidis PT4 22079, Enteritidis PT13a 21046 (PT13a-wt), Enteritidis 13a 21027 (PT13a-bf), Typhimurium

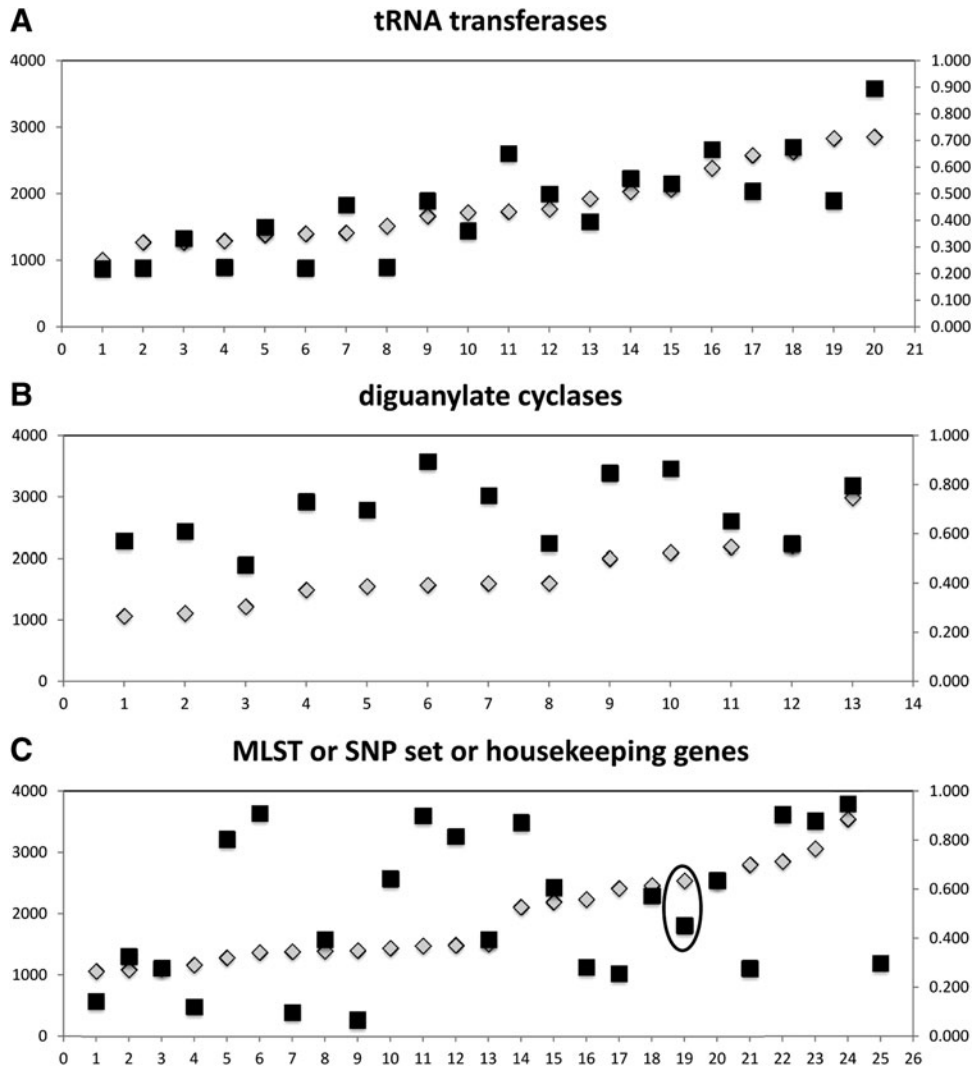

FIG. 1. Determination of mutation indices for different classes of genes of *Salmonella enterica* subspecies I by metadata analysis of all available sequences at NCBI. Dates of data acquisition spanned from August to September of 2015. Gene identity correlating to numbers listed on the *x* axes and other information about genes are listed in Table 1. *y* axis (left, gray triangles), gene length in base pairs (gray triangles). *y* axis (right, black squares), calculated values for nonsynonymous/total SNPs per gene (NS/T mutation index). Graphing results are shown for 1**A**) tRNA transferases, 1**B**) diguanylate cyclases, and 1**C**) MLST, SNP set for housekeeping genes. The circled datapoints in **(C)** indicate placement for *cyaA*. Datapoints shown as black squares plot the calculated number for NS/T mutation indices. All sequences were recovered from NCBI by BLAST using gene sequences from NC_001397.1 (McClelland *et al.*, 2001). NCBI, National Center for Biotechnology Information; NS/T, nonsynonymous to total; MLST, multilocus sequence typing; SNP, single-nucleotide polymorphism.

LT2, and Kentucky CDC191 (Guard *et al.*, 2012). These strains and the other 25 *S. enterica* poultry-associated isolates shown in Table 2 were maintained according to previously described protocols (Guard *et al.*, 2012). Of the serovars that were poultry associated and used to validate *cyaA*-targeted ASPE, serovars Typhimurium, Enteritidis, Newport, and Heidelberg are the first through fourth most common isolated from people (CDC-NCZEID, 2013). Serovars Montevideo, Schwarzengrund, and Agona often cause disease in people and have respective rankings of 7th, 23rd, and 30th (CDC-NCZEID, 2013). Serotype Kentucky is at most an infrequent cause of human disease, but there is concern that it carries a transmissible plasmid encoding antibiotic resistance (Le Hello *et al.*, 2013). Serovars Gallinarum and Pullorum do not cause human disease, but they are closely related to serovar Enteritidis and are important avian pathogens that are subject to regulatory activities (Feng *et al.*, 2013). Together, the isolates that were recovered in association with poultry and used here to validate ASPI cover a broad range of serovars and pathotypes.

Cell cultures were revived from frozen stock on brilliant green agar (Neogen, Lansing, MI) overnight at 37°C. One colony was transferred to brain-heart infusion broth (Neogen) and grown overnight at 37°C with shaking at 150 rpm. DNA was isolated from 1 mL of culture diluted to an optical density of 1.0 at wavelength = 600 nm with the PureLink Genomic DNA Mini Kit (Invitrogen, Grand Island, NY) following the manufacturer's instructions, including the RNase digestion step. Genomic DNA, 50 ng, was used as the template for the initial *cyaA* PCR. The *cyaA* PCR, ASPE hybridization, and

TABLE 1. DETAILS OF GENES INCLUDED IN ASSESSMENT OF A *cyaA*-BASED MICROSPHERE-BASED FLUIDICS ASSAY FOR ANALYSIS OF *SALMONELLA ENTERICA* SUBSPECIES I[a]

Gene ID as numbered in Figure 1	*Common name of gene*	*Gene used for BLAST*[b]	*Gene length in base pairs*	*Total DNA sequences retrieved*[c]	*Number of unique DNA sequences (T)*	*Number of unique amino acid sequences (NS)*	*Mutation index (NS/T)*	*Date accessed*	*Other information*
Figure 1A: tRNA transferases									
1	*trpS*	STM3481	1004	382	32	7	0.219	31-Aug	Tryptophan
2	*tyrS*	STM1449	1274	418	36	8	0.222	31-Aug	Tyrosine
3	*hisS*	STM2522	1274	399	36	12	0.333	31-Aug	Histidine
4	*serS*	STM0963	1293	412	40	9	0.225	31-Aug	Serine
5	*cysS*	STM0537	1386	408	40	15	0.375	31-Aug	Cysteine
6	*asnC*	STM1000	1400	412	36	8	0.222	31-Aug	Asparagine
7	*gltX*	STM2415	1416	401	37	17	0.459	31-Aug	Glutamate
8	*lysS*	STM3040	1518	394	40	9	0.225	31-Aug	Lysine
9	*glnS*	STM0686	1667	407	38	18	0.474	31-Aug	Glutamine
10	*proS*	STM0242	1719	393	36	13	0.361	31-Aug	Proline
11	*argS*	STM1909	1734	416	43	28	0.651	31-Aug	Arginine
12	*aspS*	STM1901	1773	417	40	20	0.500	31-Aug	Aspartate
13	*thrS*	STM1333	1928	433	38	15	0.395	31-Aug	Threonine
14	*metG*	STM2155	2034	398	43	24	0.558	17-Aug	Methionine
15	*glyS*	STM3655	2069	377	39	21	0.538	18-Aug	Glycine (beta subunit)
16	*pheT*	STM1338	2388	404	45	30	0.667	17-Aug	Phenylalanine (beta subunit)
17	*leuS*	STM0648	2582	400	49	25	0.510	17-Aug	Leucine
18	*alaS*	STM2827	2631	390	37	25	0.676	31-Aug	Alanine
19	*ileS*	STM0046	2835	382	40	19	0.475	17-Aug	Isoleucine
20	*valS*	STM4475	2855	393	48	43	0.896	31-Aug	Valine
Figure 1B: DGCs									
1	—	STM4551	1065	402	42	24	0.571	16-Sep	DGCs produce two diphosphate and one cyclic di-GMP molecules from guanosine triphosphate; contains the amino acid motif GGDEF, which constitutes the domain of the DGC active site. Cyclic di-GMP is an intracellular second-messenger molecule involved in biofilm formation by bacteria. DGCs are repeated many times in the genomes of pathogenic bacteria, and *Salmonella* has a total of 17 copies. The ones that are at least 1000 bp are included in this study.
2	*adrA*	STM0385	1113	402	41	25	0.610	16-Sep	
3	*yfiN*	STM2672	1221	396	40	19	0.475	16-Sep	
4	*yeaJ*	STM1283	1494	447	52	38	0.731	16-Sep	
5	*ylab*	STM0468	1551	413	43	30	0.698	16-Sep	
6	—	STM0343	1574	401	38	34	0.895	16-Sep	
7	*yjcC*	STM4264	1602	402	41	31	0.756	16-Sep	
8	—	STM1827.S	1602	448	46	27	0.562	16-Sep	
9	*yhjK*	STM3615	2006	374	33	28	0.848	16-Sep	
10	—	STM3388	2100	367	52	45	0.865	16-Sep	
11	*yfeA*	STM2410	2190	388	43	28	0.651	16-Sep	
12	—	STM2503	2214	389	50	28	0.560	16-Sep	
13	*yegE*	STM2123	2991	398	49	39	0.796	16-Sep	

(*continued*)

TABLE 1. (CONTINUED)

Gene ID as numbered in Figure 1	*Common name of gene*	*Gene used for BLAST*[b]	*Gene length in base pairs*	*Total DNA sequences retrieved*[c]	*Number of unique DNA sequences (T)*	*Number of unique amino acid sequences (NS)*	*Mutation index (NS/T)*	*Date accessed*	*Other information*
Figure 1C: Single copy genes referenced for use an MLST or housekeeping genes									
1	*recA*	STM2829	1062	398	35	5	0.143	14-Sep	Homologous recombination
2	*aroC*	STM2384	1086	405	43	14	0.326	14-Sep	Chorismate synthesis
3	*dnaN*	STM3837	1101	389	36	10	0.278	14-Sep	DNA polymerization
4	*pgk*	STM3069	1164	394	33	4	0.121	15-Sep	Glycolysis
5	*aroA*	STM0978	1284	441	46	37	0.804	14-Sep	Tryptophan biosynthesis
6	*manB*	STM2104	1371	411	66	60	0.909	14-Sep	Colanic acid synthesis
7	*atpD*	STM3865	1383	385	31	3	0.097	15-Sep	ATP synthesis
8	*ydjN*	STM1320	1392	445	48	19	0.396	15-Sep	Thiamine biosynthesis
9	*glnA*	STM4007	1401	388	30	2	0.067	14-Sep	Glutamine synthesis
10	*cysN*	STM2934	1440	395	42	27	0.643	15-Sep	Cysteine biosynthesis
11	*mocR*	SEN3898	1476	297	10	9	0.900	15-Sep	Serovar Enteritidis specific
12	*gltB*	STM3330	1486	393	54	44	0.815	14-Sep	Glutamate synthesis
13	*putP*	STM1125	1509	440	48	19	0.396	15-Sep	Serovar Enteritidis specific
14	*foxA*	STM0364	2109	403	47	41	0.872	15-Sep	Iron utilization
15	*fhuA*	STM0191	2190	70	28	17	0.607	15-Sep	Iron utilization
16	*relA*	STM2956	2235	389	39	11	0.282	31-Aug	(p)ppGpp synthesis
17	*gyrB*	STM3835	2415	378	43	11	0.256	31-Aug	Negative supercoiling of DNA
18	*thrA*	STM0002	2463	384	47	27	0.574	31-Aug	Amino acid biosynthesis
19	*cyaA*	STM3939	2540	378	42	19	0.452	31-Aug	Forms cyclic AMP
20	*nirB*	STM3474	2544	377	44	28	0.636	31-Aug	Reduction of nitrite
21	*sucA*	STM0736	2802	408	47	13	0.277	31-Aug	Oxoglutarate dehydrogenase
22	*hypB*	STM2855	2855	381	42	38	0.905	31-Aug	Nickel ligand
23	*ttrA*	STM1383	3063	448	66	58	0.879	14-Sep	Tetrathionate reduction
24	*recB*	STM2994	3546	390	39	37	0.949	31-Aug	Cleavage of DNA
25	*rpoB*	STM4153	4029	380	57	17	0.298	31-Aug	DNA-dependent RNA polymerase

[a]Reference genome was *Salmonella enterica* subspecies I serovar Typhimurium strain LT2 unless otherwise noted (NC_003197.1) (McClelland *et al.*, 2001).

[b]BLAST was done using Geneious software interface with NCBI to retrieve a minimum of 500 sequences; only *S. enterica* subspecies I sequences were included for analysis, and only if it had a complete gene. The multiple align function was used to identify unique DNA sequences. Sequences were translated and then analyzed again by the multiple align algorithm to identify the number of unique protein sequences.

[c]As obtained from NCBI.

DGC, diguanylate cyclases; MLST, multilocus sequence typing; NCBI, National Center for Biotechnology Information; NS/T, nonsynonymous to total.

Table 2. *cyaA* Single-Nucleotide Polymorphism Serotyping of Poultry-Related Environmental *Salmonella enterica* Isolates

Salmonella enterica *ISR serovar*	*Serogroup (O:H1:H2)*	*Strain ID*	*Poultry source*	*SNP expected*	*SNP(s) detected*	*Accurate detection?*
Agona	B (4,12:f,g,s:-)	26080	Fecal Dropping	None	None	Yes
Cerro	K (18:z_4,z_{23}:[z_{45}])	26034	Fecal Dropping	None	None	Yes
Enteritidis	D_1 (1,9,12:g,m:-)	100723.10	Processing Scalder Tank Foam	Enteritidis PT13a-wt	Enteritidis PT13a-wt	Yes
Enteritidis	"	22085	Layer Egg	Enteritidis PT13a-wt	Enteritidis PT13a-wt	Yes
Enteritidis	"	100723.09	Broiler Carcass Rinse	Enteritidis PT13a-wt	Enteritidis PT13a-wt	Yes
Gallinarum	D_1 (1,9,12:-:-)	99117	Chicken House	None	None	Yes
Heidelberg	B ([1],4,[5],12:r:1,2)	25023	Chicken House	None	Typhimurium	No[a]
Heidelberg	"	100304.43	Broiler Carcass Rinse	None	Typhimurium	No[a]
Heidelberg	"	100709.09	Processing Scalder Tank Foam	None	Typhimurium	No[a]
Kentucky	C_2C_3 ((8),(20):i:z_6)	26031	Fecal Dropping	Kentucky	Kentucky	Yes
Kentucky	"	100304.7	Broiler Carcass Rinse	Kentucky	Kentucky	Yes
Kentucky	"	100304.8	Processing Scalder Tank Foam	Kentucky	Kentucky	Yes
Kentucky	"	101116.01	Broiler Carcass Rinse	Kentucky	Kentucky	Yes
Kentucky	"	101116.03	Processing Scalder Tank Water	Kentucky	Kentucky	Yes
Kentucky	"	100709.01	Broiler Carcass Rinse	Kentucky	Kentucky	Yes
Montevideo	C_1 (6,7:g,m,s:-)	26059	Fecal Dropping	None	None	Yes
Newport	C_2 (6,8:e,h:1,2)	26042	Fecal Dropping	None	None	Yes
Pullorum	D_1 (9,12:-:-)	99113	Chicken House	None	None	Yes
Schwarzengrund	B (1,4,12,27:d:1,7)	100723.15	Processing Scalder Tank Water	None	None	Yes
Typhimurium	B (1,4,5,12:i:1,2)	100304.53	Processing Scalder Tank Water	Typhimurium	Typhimurium	Yes
Typhimurium	"	100616.91	Broiler Carcass Rinse	Typhimurium	Typhimurium	Yes
Typhimurium	"	100616.89	Broiler Carcass Rinse	Typhimurium	Typhimurium	Yes
Typhimurium var. Copenhagen	B (1,4,12:i:1,2)	26020	Fecal Dropping	Typhimurium	Typhimurium	Yes
Typhimurium var. Copenhagen	"	100304.32	Broiler Carcass Rinse	Typhimurium	Typhimurium	Yes
Typhimurium var. Copenhagen	"	100304.63	Processing Scalder Tank Foam	Typhimurium	Typhimurium	Yes

[a]Serovars Heidelberg and Typhimurium were differentiated by SNPs in the ISR region.
ISR, intergenic sequence ribotyping; SNP, single-nucleotide polymorphism.

SNP detection protocols were used as described for subtyping of all *S. enterica* isolates.

PCR assay design for gene cyaA

Entire *cyaA* genes (~ 3 Kb) for the five *S. enterica* reference strains were retrieved from GenBank (Benson *et al.*, 2013) and aligned using the MEGA 5.0 software package (Tamura *et al.*, 2011). The resultant alignment file was truncated to a 300-basepairs (bp) region (bp 1900–2199 in STM3939) that was found to contain descriptive SNPs. General PCR *cyaA* primers were designed against conserved regions among all five reference strains: SAL-*cyaA*-F1 (5′-CCGGATAGCGTGGAGG TGTT-3′) and SAL-*cyaA*-R2 (5′-CACCACTGACGGCAATT TCACC-3′). The thermocycler used was a Realplex 4S (Eppendorf, Hauppauge, NY). The optimized *cyaA* PCR consisted of 50 ng DNA, AccuStart PCR 2× ToughMix (Quanta Biosciences, Gaithersburg, MD), and 400 nM each SAL-cya-F1 and SAL-cya-R2 primers (Biosearch, Novato, CA). The PCR program was 95°C for 10 min; 35 cycles of 95°C for 20 s, 60.6°C for 30 s, 72°C for 1 min; 72°C for 10 min.

To determine the specificity of the *cyaA* PCR assay to *S. enterica*, genomic DNA was extracted from a panel of negative controls (*Campylobacter lari* strain 43675, *Campylobacter coli* strain 33559, *Campylobacter jejuni* strain 14544, *Escherichia coli* strain EDL 933, and *L. monocytogenes* strain Li23). To determine the limit of detection for each reference strain, *cyaA* amplicons were generated using 0.001 pg–50 ng of template genomic DNA. For both the specificity and limit of detection tests, the *cyaA* PCR protocol described above and the hybridization and detection protocols described below were used as described.

ASPE primer design, cyaA amplicon hybridization, and SNP detection

The initial *cyaA* PCR assay used final primer concentrations = 400 nM, Tm = 60.6°C to produce amplicons for the ASPE reaction at genomic DNA template concentrations ≥0.001 ng per *cyaA* PCR. The ASPE primers (Table 3) were designed to contain both the reverse complement of the ANTI-TAG sequence attached to the MagPlex-TAG-coupled microspheres (Luminex), and a *cyaA* gene sequence with the 3′ terminal end representing the discriminatory SNP. Primers were designed for both the SNP and wild-type sequence for the reference *S. enterica* strains, and each primer was coupled to a unique MagPlex-TAG-coupled microsphere to perform multiplexed ASPE.

Using the *cyaA* amplicons from the initial PCR step, 5 μL of that PCR was mixed with 2 μL ExoSAP-IT reagent (Affymetrix, Santa Clara, CA) and incubated at 37°C for 30 min, then 80°C for 15 min. The ASPE reaction was performed using 0.75 U Tsp DNA polymerase, ASPE reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.25 mM $MgCl_2$, 5 μM each of dATP, dTTP, dGTP, 5 μM biotin-14-dCTP, 25 nM each TAG-ASPE primer, and 5 μL ExoSAP IT-treated PCR template amplicon. All reagents used were from Invitrogen. The ASPE cycling program consisted of 96°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 1 min, 74°C for 2 min.

MagPlex-TAG-coupled microspheres were supplied by the manufacturer at a concentration of 2.5×10^6 microspheres/mL. A hybridization mixture was made of 1 μL of each of the eight MagPlex-TAG-coupled microspheres (equal to 2500 microspheres of each), 17 μL 2× Tm Hybridization Buffer (0.2 M Tris-HCl, pH 8.0, 0.4 M NaCl, 0.16% Triton X-100), 5 μL ASPE reaction, and 20 μL PCR water for a final volume of 50 μL. The hybridization was carried out at 96°C for 90 s and then 37°C for 30 min. The hybridized microspheres were pelleted by a magnetic separator (Perkin Elmer, Shelton, CT), washed with Tm Hybridization Buffer, and incubated with streptavidin-R-phycoerythrin (Invitrogen) at a final concentration of 2 μg/mL in 75 μL Tm Hybridization Buffer at 37°C for 15 min. Fifty microliters were analyzed at 37°C in the MAGPIX instrument (Luminex).

Sample data were analyzed using the xPONENT® ver. 4.2 software package (Luminex), and positive and negative signals for each microsphere bead pair (SNP and wild type) were determined in the following manner. The average median fluorescence intensity (MFI) of the two no template control (NTC) wells was subtracted from the MFI of a sample to yield the net MFI for each sample well. For each bead pair, a net MFI from the non-SNP-associated bead that was at least 30% higher than the SNP-associated bead indicated a negative signal for that SNP. Conversely, 30% higher from the SNP bead than the non-SNP bead indicated a positive for the SNP. A background sample consisted of a NTC from the PCR that had undergone ASPE and hybridization.

Validation of cyaA-targeted ASPE for analyzing S. enterica

The set of five *Salmonella* reference strains was tested in various combinations to determine the specificity of the method. Genomic DNA of a single strain was used as input to the initial PCR, followed by ASPE with one set of primers (one set of two primers, one primer for each allele) and hybridization with the two corresponding MagPlex-TAG-coupled microspheres; each strain was tested in this manner. First, 50 ng of genomic DNA of a single strain was used as input to the initial PCR, followed by ASPE with every possible combination of the four primer sets (two to four sets in various combinations) and hybridization with the corresponding MagPlex-TAG-coupled microspheres; each strain was tested in this manner. Finally, genomic DNA from the reference strains was mixed in every possible combination of two to five strains. For these combinations, two series were done: in one, the initial input to the PCR was 50 ng of each strain, regardless of the number of strains, and in another, the total input was 50 ng (two strains at 25 ng/strain, three strains at 16.7 ng/strain, four strains at 12.5 ng/strain, or five strains at 10 ng/strain). The resulting PCR amplicons from these combinations were used in ASPE reactions with all four primer sets and hybridization with all eight MagPlex-TAG-coupled microspheres. The panel of 25 *S. enterica* isolates encompassing 12 serotypes was analyzed using the optimized *cyaA* SNP method (Table 2). Genomic DNA was extracted from these isolates as explained before in the PCR Assay Design for Gene *cyaA* section.

Results

Review of cyaA SNPs present in the NCBI database

BLAST search using sequence from *Salmonella enterica* serotype Typhimurium LT2 (NC_003197.1) *cyaA* gene STM3939 recovered 378 sequences of *cyaA* from NCBI

Table 3. Primer Information for Single-Nucleotide Polymorphism-Targeted Allele-Specific Primer Extension Hybridization Reactions Targeted to *cyaA* of *Salmonella enterica*

Reference strain target	*Primer name*	*Anti-TAG sequence on MagPlex®-TAG™ microsphere (5′ to 3′)*	*ASPE primer sequence (5′ to 3′)*[a]	*Wild-type base*	*SNP base*
Enteritidis PT13a-wt	ASPE-A013-C120-S16	AGTGAATGTAAGATTAT GTATTTG	**CAAATACATAATCTTACATTCACT**CA CCCGTCAGGAGACC	C	
	ASPE-A20-A162-S14	TATTGTTGAATGTGTTTA AAGAGA	**TCTCTTTAAACACATTCAACAATA**CA CCCGTCAGGAGACT		T
Enteritidis PT13a-bf	ASPE-A026-A206-S17	TTTGATTTAAGAGTGTTG AATGTA	**TACATTCAACACTCTTAAATCAAA**GT GCAGAAGCTGGAGAA	A	
	ASPE-A033-C225-S19	GTAAGAGTATTGAAATTA GTAAGA	**TCTTACTAATTTCAATACTCTTAC**GTG CAGAAGCTGGAGAG		G
Typhimurium	ASPE-A047-T120-S16	AAATTAGTTGAAAGTATG AGAAAG	**CTTTCTCATACTTTCAACTAATTT**GGC AGACGTGGGGA	A	
	ASPE-A57-G162-S14	AGAGTATTAGTAGTTATT GTAAGT	**ACTTACAATAACTACTAATACTCT**GGC AGACGTGGGGG		G
Kentucky	ASPE-A066-G206-S17	TATTAGAGTTTGAGAATA AGTAGT	**ACTACTTATTCTCAAACTCTAATA**CGC TATCGAATTCTACGGC	C	
	ASPE-A076-A225-S19	AAAGAATTAGTATGATAG ATGAGA	**TCTCATCTATCATACTAATTCTTT** CGC TATCGAATTCTACGGA		A

[a]Bold portion of the primer represents the TAG sequence for the primer to hybridize to the microsphere, while the nonbolded portion represents the allelic portion ending in the discriminatory SNP (underlined base).

ASPE, allele-specific primer extension; SNP, single-nucleotide polymorphism.

Table 4. Other Single-Nucleotide Polymorphisms in the Adenylate Cyclase *cyaA* Gene of *Salmonella enterica* that Can Be Combined with *dkgB*-Linked Intergenic Sequence Ribotyping to Resolve Some Serotypes and to Identify Some Subtypes[a,b,c]

Nucleotide number in cyaA	*Allele 1*	*Allele 2*	*Nucleotides between SNPs*	*Nucleotide number in cyaA*	*Allele 1*	*Allele 2*	*Nucleotides between SNPs*	*Nucleotide number in cyaA*	*Allele 1*	*Allele 2*	*Nucleotides between SNPs*	*Nucleotide number in cyaA*	*Allele 1*	*Allele 2*	*Nucleotides between SNPs*
261	c	t	—	960	g	a	–75	1621	a	c	–46	**2016**	**g**	**a**	**–12**
277	c	t	–16	1023	t	c	–63	1737	c	t	–116	***2019***	***c***	***t***	***–3***
291	c	t	–14	1032	a	t	–9	1831	a	c	–94	**2025**	**c**	**a**	**–6**
321	c	t	–30	1062	a	g	–30	1875	g	c	–44	**2061**	**r**	**a**	**–36**
333	c	t	–12	1089	g	a	–27	1911	s	g	–36	**2124**	**c**	**t**	**–63**
381	y	t	–48	1146	y	t	–57	1914	r	g	–3	**2127**	**g**	**t**	**–3**
465	g	a	–84	1176	a	g	–30	**1923**	**c**	**t**	**–9**	2182	a	g	–55
588	g	a	–123	1227	t	c	–51	**1932**	**g**	**a**	**–9**	2223	y	c	–41
597	g	c	–9	1272	g	c	–45	**1944**	**t**	**c**	**–12**	2250	c	t	–27
603	t	c	–6	1275	t	g	–3	**1945**	**c**	**t**	**–1**	2319	r	a	–69
657	t	c	–54	1335	c	t	–60	**1953**	**c**	**t**	**–8**	2352	t	c	–33
681	c	t	–24	1374	c	t	–39	**1959**	**c**	**t**	**–6**	2364	g	a	–12
705	a	g	–24	1404	c	t	–30	**1968**	**a**	**g**	**–9**	2382	c	t	–18
783	g	a	–78	1422	t	c	–18	**1974**	**c**	**t**	**–6**	2397	t	c	–15
802	c	t	–19	1425	c	t	–3	**1980**	**a**	**g**	**–6**	2409	t	c	–12
813	c	c	–11	1434	c	t	–9	**1983**	**t**	**c**	**–3**	2442	c	t	–33
825	t	c	–12	1437	t	c	–3	**1986**	**c**	**t**	**–3**	2453	m	a	–11
828	c	t	–3	1471	c	t	–34	**1989**	**G**	**a**	**–3**	2454	g	a	–1
850	g	t	–22	1551	g	a	–80	**1992**	**A**	**g**	**–3**	2475	g	a	–21
852	t	c	–2	1560	g	a	–9	**1995**	**T**	**c**	**–3**	2482	a	g	–7
885	a	g	–33	1575	c	t	–15	**2004**	**C**	**t**	**–9**	2487	c	t	–5

[a]DNA nucleotide code: g, guanosine; a, adenosine; t, thymidine; c, cytidine; w, a or t, weak (two H-bonds); s, c or g, strong (three H-bonds); m, a or c, aMino; y, c or t, pYrimidine; r, g or a, puRine.

[b]SNPs in bold are within the coding region for cyaA flanked by assay primers F1 and R1; the SNP in italics at bp 2019 differentiates phage type lineages of serovar Enteritidis; the PT4 lineage (NC_011294) has a C, PT13a/8/14b lineage has a T (NZ_CP007175).

[c]An unlisted SNP at bp 2105 further distinguishes serovar Enteritidis wild-type PT13a and PT4 from a biofilm forming strain of PT13a. The first two have an adenosine (a) and the latter has a cytidine (c) (Morales *et al.*, 2007; Guard *et al.*, 2011).

SNP, single-nucleotide polymorphism.

(Line 64 in S1). The set of *cyaA* sequences available was heavily weighted toward two serotypes. Of the 378 sequences in the set as of August 2015, 175 (46.3%) were from serotype Enteritidis and 116 (30.7%) were from serotype Paratyphi A. Other serotypes with more than three entries included serotypes Typhimurium with 21 (5.6%), Newport with 12 (3.2%), and Heidelberg with 5 (1.3%). All other serotypes had fewer than five representative strains. Percent GC content of *cyaA* within *S. enterica* subspecies I ranged from 54.2% to 54.7%, percent identical sites ranged from 98.6% to 100%, and reported gene lengths were from 2543 to 2547 bp. Gene length depended on inclusion of terminating codons, and thus, all sequences were trimmed to a length of 2540 bp. Among the 378 sequences meeting parameters of the *cyaA* BLAST, 42 DNA sequences were unique, and these translated into 19 unique amino acid sequences. The NS/T change thus gave a mutation index of 19/42 or 0.452 (Fig. 1). In other words, a little less than one amino acid was altered for approximately every two differences in nucleotides.

To compare the NS/T of *cyaA* to other genes, a set of 20 tRNA transferases greater than 1000 bp was analyzed, and there was one gene analyzed per common amino acid. This set of genes was chosen because it was hypothesized to be highly evolved and thus likely to have a fairly stable NS/T mutation index. Figure 1A indicates that the NS/T index may be impacted by the size of the gene, so results are listed relative to increasing gene size. For tRNA transferases between 1000 and 2000 bp, the average NS/T index was 0.359 and the standard deviation was 0.1421. For genes between 2000 and 3000 bp, the average NS/T index was 0.617 and the standard deviation was 0.1444. Thus, NS/T indices for the tRNA transferases are an example of SNP variation increasing proportionately to gene size. The five tRNA transferases least likely to generate amino acid sequence variation were *serS*, *asnC*, *lysS*, *leuS*, and *ileS*, and the entire class had an average NS/T index of 0.449. The NS/T index for *cyaA* of 0.452 suggests that *cyaA* has an amino acid sequence about as stable as that of an average tRNA transferase.

Results from analysis of the DGC differed (Fig. 1B). For genes between 1000 and 2000 bp, the average NS/T index was 0.662 and the standard deviation was 0.1337, whereas respective values for genes greater than 2000 bp were 0.744 and 0.1329. Although NS/T indices for shorter versus longer genes for tRNA transferases were significantly different ($p = 0.0009$), the same parameter for the DGC genes was not ($p = 0.3055$). These results suggest that DGC genes of *S. enterica* subspecies I are significantly more likely to generate amino acid sequence variation than the tRNA transferases, regardless of gene size ($p = 0.0003$). All of the genes in the DGC class appear to undergo significantly frequent amino acid substitution in comparison to the tRNA transferases.

The third set of genes analyzed was chosen with no emphasis on relatedness of function, but they were used in other genomic investigations. The *cyaA* gene was included in this set (Fig. 1C, circled datapoint). Results indicate that NS/T indices did not differ significantly according to the length of the gene ($p = 0.2907$). The standard deviation in NS/T indices for this third set of genes was 0.299, whereas it was 0.185 and 0.134 for tRNA transferases and DGC genes, respectively. Twelve genes in set 3 had NS/T indices less than *cyaA*, and 12 genes had NS/T indices that were greater (Table 1). These results suggest that random selection of genes is likely to generate some variation between selected gene targets that is due to innate differences in mutation index. The gene *cyaA* appears to be located at a midpoint of variation.

Accuracy and sensitivity of cyaA SNP detection by ASPE for S. enterica *strains*

For the four SNP-containing reference strains (Enteritidis PT13a-wt, Enteritidis PT13a-bf, Typhimurium, Kentucky), all possible combinations (from singleplex to five-plex) yielded expected SNP patterns and always matched the actual patterns determined by the assay with 100% accuracy. The fifth strain, Enteritidis PT4, was used as a negative control since it does not possess an SNP within this 300-bp region of the *cyaA* gene. However, Table 4 shows the large number of SNPs that could detect Enteritidis PT4 and other phage types as a positive reaction in future assays. The 100% SNP pattern detection accuracy did not change based on the two different ways in which the template combinations were created (50 ng DNA for each strain or 50 ng DNA total). These results highlight the specificity and accuracy of *cyaA* SNP detection using this optimized assay against the reference strains used to develop the ASPE primers and MagPlex-TAG magnetic microspheres.

Validation of ASPE for poultry-related Salmonella enterica *serotype Enteritidis environmental isolates*

The *cyaA* SNP assay demonstrated high agreement between the expected and actual SNP pattern observed for the different serotypes within the environmental isolate panel (89%; Table 2). It was expected that serotype Heidelberg would not be differentiated from serovar Typhimurium, because it lacked an SNP in the region under investigation. As expected, poultry-related serotypes not used to design the assay (Heidelberg, Agona, Cerro, Gallinarum, Infantis, Montevideo, Newport, Pullorum, Schwarzengrund) did not have SNPs for any of the target bases. However, review of available *cyaA* sequences revealed that many other SNPs could be used to target other serotypes (Table 4). Two pathotypes of Phage Type 13a serotype Enteritidis, namely the egg-contaminating strain (PT13a-wt 21046) and a biofilm-forming non-egg contaminating strain (PT13a-bf 21027), had discriminatory SNPs in the *cyaA* gene (Morales *et al.*, 2007). When used to group a panel of poultry-related environmental Enteritidis isolates, the correct SNP pattern was found for the two known subtypes of PT13a. PT13a and PT8 belong in the same evolutionary lineage and vary by plasmid content, so they would be expected to group together (Threlfall *et al.*, 1993; Liebana *et al.*, 2004). One PT14b grouped with the PT13a wt strain 21046, which is a finding supported as correct by results from NCBI bioproject 219482 (Rehman *et al.*, 2014). As expected, two PT4 isolates did not have either of the SNPs associated with the PT13a/PT8 lineage (Thomson *et al.*, 2008). These results indicated that the *cyaA* SNP assay worked well for distinguishing between previously characterized PT13a pathotypes and could distinguish the PT4 lineage from PT13a/8.

Conclusions

These analyses suggest that *cyaA* SNPs targeted by ASPE will support and extend the use of ISR applied as a screening

method for assigning serotype to *S. enterica*. It will help distinguish between serovars that might share the same ISR sequence (e.g., ISR group UN0006), provide some information about phage type and pathotype, and ultimately achieve some subtyping within serotype. An incidental finding is that tRNA transferases and *cyaA* give a conservative assessment of subtype in comparison to DGC genes. Thus, DGC genes might be most valuable for identifying strains rapidly emerging within subtypes even within a single outbreak. Given the ability of the Luminex MagPlex system to identify up to 150 custom beads within a single well, assays can be developed and optimized to detect up to 75 different SNPs. Thus, finding at least 84 SNPs across a gene that is 2540 bp suggests that *cyaA* is an ideal target for assay development. Cost per sample for bead-based capillary systems has been quoted to range from $40 to $50 per sample, but the ability to process multiple SNPs within single wells might make it an affordable confirmatory assay for properly equipped laboratories. Other methods that do not require specialized equipment or intensive maintenance regimens, such as ISR, appear less costly for conducting routine screening for serotype and field investigations of on-farm ecology (Guard *et al.*, 2012; Jean-Gilles Beaubrun *et al.*, 2014).

Comparative whole genome analyses have revealed a large number of potentially discriminatory SNPs among many different genes (Zheng *et al.*, 2014). In this instance, SNP analysis was done within the context of how likely *cyaA* is to mutate compared to different sets of genes, and it was assessed for the number of target sites it has that are amenable to analysis by xMAP technology. As information is assessed by whole genome analysis from hundreds of strains, target sites will be identified that provide optimal genomic information about sources of outbreaks that will facilitate protecting the safety of the food supply.

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Authors' Contributions

J.G. designed and implemented initial research, M.J.R. designed, developed, and conducted fluids experimentation, and J.G. and M.J.R. wrote the article. Z.A., S.O.B., and P.K. provided statistical oversight and some bioinformatics support.

Disclosure Statement

No competing financial interests exist.

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