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ORIGINAL ARTICLE

Conserved CRISPR arrays in *Salmonella enterica* serovar Infantis can serve as qPCR targets to detect Infantis in mixed serovar populations

A.K. Richards[1], B.A. Hopkins[2] and N.W. Shariat[1]

1 Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA, USA
2 International Technical Animal Production and Processing Solutions (iTAPPS), Overland Park, KS, USA

Significance and Impact of the Study: The incidence of human salmonellosis cases caused by *Salmonella enterica* serovar Infantis (ser. Infantis) has been increasing, as has its prevalence in broiler chickens, which are a frequent reservoir of *Salmonella*. A cluster of ser. Infantis genetically linked to an outbreak strain have been identified in numerous processing facilities. A qPCR assay targeting CRISPR elements that are unique to ser. Infantis has been developed and can detect this serovar directly from mixed cultures. This assay is sensitive enough to reveal ser. Infantis within a mixed *Salmonella* population where it constitutes only 0·1% of the population. The rapid nature of qPCR lends this assay to high-throughput screening of poultry samples to detect this important pathogen.

Keywords
CRISPR, food safety, Infantis, poultry, qPCR, *Salmonella*, surveillance.

Correspondence
Nikki W. Shariat, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA.
E-mail: nikki.shariat@uga.edu

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Abstract

Salmonellosis is a leading bacterial cause of foodborne illness, and numerous *Salmonella enterica* serovars have been responsible for foodborne outbreaks. In the United States outbreaks are often linked to poultry and poultry-related products. The prevalence of *Salmonella* serovar Infantis has been increasing in poultry processing facilities over the past few years and in 2018 was identified as the causative agent for a large multistate outbreak linked to raw chicken. CRISPR-typing is a subtyping approach based on PCR and the sequencing of two *Salmonella* loci, CRISPR1 and CRISPR2. CRISPR-typing was used to interrogate 138 recent (2018–2019) isolates and genomes of ser. Infantis. Results show that the CRISPR elements are remarkably conserved in this serovar. The most conserved spacers, and those also unique to ser. Infantis, were used as targets to develop a ser. Infantis-specific qPCR assay. This assay was able to detect ser. Infantis in mixed serovar cultures of *Salmonella*, down to 0·1% of the population, highlighting the utility of this molecular approach in improving surveillance sensitivity for this important food safety pathogen.

Introduction

Salmonella enterica is a leading bacterial pathogen responsible for foodborne illness in the United States, causing over one million estimated illnesses each year (Scallan *et al.* 2011; Tack *et al.* 2019). Salmonellosis is generally associated with contaminated fresh produce and food-producing animals. Poultry is a reservoir for *Salmonella*, and raw and undercooked poultry products are responsible for a number of outbreaks each year (Antunes *et al.* 2016). Ser. Infantis is one of the top serovars responsible for human illness in Europe and the United States, and is also frequently isolated from poultry (EFSA and ECDC 2018; Marder *et al.* 2018). Over the last decade, the incidence of human infections linked to ser. Infantis have increased by 50% in the United States, and the percentage of broiler samples positive for this serovar have also increased by over 50% (Marder *et al.* 2018). In 2018, an outbreak of *Salmonella* ser. Infantis that was associated with a raw frozen chicken product sickened 129 people and the outbreak strain was resistant to a number of antibiotics (CDC 2019b). Subsequent investigations and whole genome sequence analysis showed that the outbreak strain and closely related isolates were identified in

76 poultry processing plants in the United States, demonstrating the prevalence of this serovar in the poultry industry (CDC 2019b). Given the 2018 outbreak, the overall increase in this serovar from poultry processing facilities, and the rapidly increasing proportion of these isolates that are resistant to medically important antibiotics (United States Food and Drug Administration 2019), there is a need for diagnostic tools to be able to rapidly detect ser. Infantis with greater sensitivity and accuracy.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) are present in approximately 45% of all sequenced bacterial genomes (Grissa *et al.* 2007). CRISPR arrays consist of conserved direct repeat sequences that are interspaced with highly variable spacer sequences. Different strains of bacteria often harbour different spacer sequences, and due to this dynamic property, CRISPR-typing is an effective way to serotype and subtype bacteria, including *Salmonella* (Shariat and Dudley 2014). In *Salmonella*, there are two CRISPR loci, CRISPR1 and CRISPR2 and the direct repeats are 29 nucleotides long, while the spacers are typically 32 nucleotides long (Touchon and Rocha 2010). Sequence comparisons of CRISPR spacer content can be used to discriminate between different *Salmonella* strains (e.g. Liu *et al.* 2011; Fabre *et al.* 2012; Shariat *et al.* 2013b, 2013c; Bachmann *et al.* 2014; Almeida *et al.* 2017; Vosik *et al.* 2018; Rauch *et al.* 2018). Conserved CRISPR spacers within a *Salmonella* serovar can also be used to generate effective molecular diagnostic tools for both serotyping (Fabre *et al.* 2012, 2014; Bugarel *et al.* 2018) and also to reveal mixed serovar populations (Thompson *et al.* 2018; Cox *et al.* 2019). The objective of this study was to determine the diversity of the CRISPR loci among a broad collection of 138 ser. Infantis isolates and genomes, and to use this information to generate a qPCR assay specific for ser. Infantis.

Results and discussion

We examined the CRISPR arrays from 138 recent (2018–2019) *Salmonella* ser. Infantis isolates or genomes (Table 1). These included five isolates collected from broiler litter, six isolates from commercial broiler carcasses, six clinical broiler isolates and 122 whole genome sequences that were publicly available on NCBI. Of these whole genome sequences, 30 were human clinical isolates from the Center for Disease Control and Prevention (CDC), and 92 were isolates from various food animal sources that were uploaded by the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS). In our sample set, we identified two CRISPR1 alleles and these differed from each other by a single spacer and we found five CRISPR2 alleles that were

Table 1 *Salmonella* serovar Infantis isolates used in this study

Isolate	Source	Sequence type
011819-1*	Veterinary—clinical	ICT 1
011819-2	Veterinary—clinical	ICT 1
012819-2	Veterinary—clinical	ICT 1
012819-3*,†	Veterinary—clinical	ICT 1
022519-13*	Poultry processing	ICT 2
022519-16	Poultry processing	ICT 2
032519-18	Poultry processing	ICT 2
040819-3	Poultry processing	ICT 2
040819-6*,†	Poultry processing	ICT 2
120518-6	Veterinary—clinical	ICT 2
120518-86	Veterinary—clinical	ICT 2
3945A	Poultry litter	ICT 2
3962B	Poultry litter	ICT 2
022519-3	Poultry processing	ICT 3
67321A	Poultry litter	ICT 5
4A732	Poultry litter	ICT 5
PDT000296987.2	Human—clinical	ICT 1
PDT000286940.2	Human—clinical	ICT 1
PDT000286272.2	Human—clinical	ICT 1
PDT000285450.2	Human—clinical	ICT 1
PDT000310894.2	Human—clinical	ICT 1
PDT000297288.2	Human—clinical	ICT 4
PDT000296770.4	Human—clinical	ICT 4
PDT000278566.2	Human—clinical	ICT 4
PDT000280593.2	Human—clinical	ICT 4
PDT000285555.2	Human—clinical	ICT 4
PDT000299917.2	Human—clinical	ICT 4
PDT000283772.2	Human—clinical	ICT 4
PDT000285520.2	Human—clinical	ICT 4
PDT000296484.2	Human—clinical	ICT 4
PDT000288071.2	Human—clinical	ICT 4
PDT000275355.2	Human—clinical	ICT 4
PDT000292739.2	Human—clinical	ICT 4
PDT000307628.2	Human—clinical	ICT 4
PDT000284024.2	Human—clinical	ICT 4
PDT000279710.2	Human—clinical	ICT 4
PDT000277686.2	Human—clinical	ICT 4
PDT000296518.2	Human—clinical	ICT 4
PDT000290258.2	Human—clinical	ICT 4
PDT000297348.2	Human—clinical	ICT 4
PDT000280657.2	Human—clinical	ICT 4
PDT000288109.2	Human—clinical	ICT 4
PDT000297468.2	Human—clinical	ICT 4
PDT000292750.2	Human—clinical	ICT 4
PDT000288112.2	Human—clinical	ICT 4
PDT000321254.1	Human—clinical	ICT 4
PDT000483442.2	Animal-cattle-dairy cow	ICT 1
PDT000375899.1	Animal-cattle-steer	ICT 1
PDT000330383.1	Animal-cattle-steer	ICT 1
PDT000315381.2	Animal-chicken-young chicken	ICT 1
PDT000467209.1	Animal-chicken-young chicken	ICT 1
PDT000384250.2	Animal-chicken-young chicken	ICT 1
PDT000280998.2	Animal-chicken-young chicken	ICT 1
PDT000483456.2	Animal-swine-market swine	ICT 1

(continued)

Table 1 (continued)

Isolate	Source	Sequence type
PDT000485704·2	Animal-swine-market swine	ICT 1
PDT000293391·2	Animal-swine-market swine	ICT 1
PDT000422143·1	Animal-swine-market swine	ICT 1
PDT000352060·1	Animal-swine-market swine	ICT 1
PDT000481159·2	Animal-swine-market swine	ICT 1
PDT000307820·2	Animal-swine-market swine	ICT 1
PDT000465240·1	Animal-swine-market swine	ICT 1
PDT000417472·2	Animal-swine-sow	ICT 1
PDT000296284·2	Animal-swine-sow	ICT 1
PDT000333685·1	Animal-swine-sow	ICT 1
PDT000340461·1	Animal-swine-sow	ICT 1
PDT000409559·1	Animal-swine-sow	ICT 1
PDT000403797·2	Animal-swine-sow	ICT 1
PDT000439588·1	Chicken carcass	ICT 1
PDT000276933·2	Chicken carcass	ICT 1
PDT000440541·2	Chicken carcass	ICT 1
PDT000387489·1	Chicken carcass	ICT 1
PDT000403645·1	Comminuted beef	ICT 1
PDT000387488·1	Comminuted beef	ICT 1
PDT000518230·1	Comminuted beef	ICT 1
PDT000355128·1	Comminuted beef	ICT 1
PDT000287526·2	Comminuted chicken	ICT 1
PDT000328447·1	Comminuted chicken	ICT 1
PDT000362918·1	Comminuted chicken	ICT 1
PDT000375928·1	Comminuted chicken	ICT 1
PDT000408964·1	Comminuted chicken	ICT 1
PDT000418668·1	Comminuted chicken	ICT 1
PDT000503142·2	Comminuted chicken	ICT 1
PDT000479733·2	Comminuted chicken	ICT 1
PDT000312443·2	Comminuted chicken	ICT 1
PDT000487670·1	Comminuted chicken	ICT 1
PDT000281009·2	Comminuted chicken	ICT 1
PDT000430835·1	Comminuted chicken	ICT 1
PDT000465138·1	Comminuted chicken	ICT 1
PDT000477148·2	Comminuted chicken	ICT 1
PDT000485808·2	Comminuted chicken	ICT 1
PDT000500602·2	Comminuted chicken	ICT 1
PDT000360864·1	Comminuted chicken	ICT 1
PDT000372284·1	Comminuted chicken	ICT 1
PDT000388817·1	Comminuted chicken	ICT 1
PDT000403438·1	Comminuted chicken	ICT 1
PDT000417358·2	Comminuted chicken	ICT 1
PDT000372327·1	Comminuted chicken	ICT 1
PDT000523099·1	Comminuted turkey	ICT 1
PDT000510882·2	Comminuted turkey	ICT 1
PDT000337182·1	Comminuted turkey	ICT 1
PDT000068542·4	Product-eggs-raw-whole	ICT 1
PDT000344101·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000409525·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000479735·2	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000285906·2	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000430586·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000468320·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000316441·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000327698·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000312444·2	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000522454·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000300014·2	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000311692·2	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000390363·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000305903·2	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000384214·1	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000509395·2	Product-raw-ground, comminuted or otherwise nonintact-pork	ICT 1
PDT000347510·1	Product-raw-intact-beef	ICT 1
PDT000393076·1	Product-raw-intact-pork	ICT 1
PDT000293855·2	Product-raw-intact-pork	ICT 1
PDT000397506·1	Product-raw-intact-pork	ICT 1
PDT000293307·4	Product-raw-intact-pork	ICT 1
PDT000372568·1	Product-raw-intact-pork	ICT 1
PDT000522024·1	Product-raw-intact-siluriformes	ICT 1
PDT000281140·2	Raw intact chicken	ICT 1
PDT000294591·2	Raw intact chicken	ICT 1
PDT000350398·1	Raw intact chicken	ICT 1
PDT000465753·1	Raw intact chicken	ICT 1
PDT000479727·2	Raw intact chicken	ICT 1
PDT000490302·2	Raw intact chicken	ICT 1
PDT000405500·1	Raw intact chicken	ICT 1
PDT000331839·1	Raw intact chicken	ICT 1
PDT000275940·2	Raw intact chicken	ICT 1
PDT000285861·2	Raw intact chicken	ICT 1
PDT000292831·2	Raw intact chicken	ICT 1
PDT000340406·1	Raw intact chicken	ICT 1
PDT000467122·1	Raw intact chicken	ICT 1
PDT000490292·2	Raw intact chicken	ICT 1

*Isolates used in ser. Infantis only qPCR assay.
†Isolates used in mixed ser. Infantis and ser. Kentucky qPCR experiments.

defined by either missing or duplicated spacers (Fig. 1). The allelic diversity of ser. Infantis CRISPR arrays is lower than we have observed in some other serovars, particularly at the CRISPR1 locus. For example, in a collection

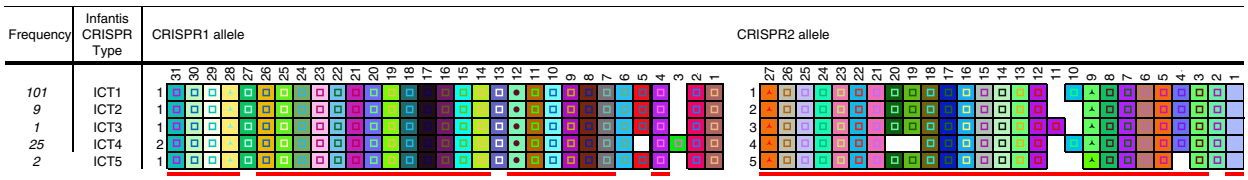

Figure 1 Serovar Infantis CRISPR arrays are highly conserved. The arrays show the different alleles identified, based on the CRISPR spacer composition. Individual spacers are shown as uniquely coloured boxes as in Horvath *et al.* (2008). The shape inside each box denotes the length of the spacer; most *Salmonella* spacers are 32 nucleotides long and are represented by a square shape. For clarity, the CRISPR direct repeat sequences are not shown. The spacers are aligned from 5′ to 3′ with gaps representing the absence of a given spacer. The solid vertical line between spacers 4 and 5 in the CRISPR1 array represents a DNA sequence made up of a truncated spacer/direct repeat hybrid. The bold red line below represents spacer sequences that are specific to ser. Infantis, when compared to a database of 130 *Salmonella* serovars. [Colour figure can be viewed at wile yonlinelibrary.com]

of 161 ser. Enteritidis isolates, we found seven alleles each for CRISPR1 and for CRISPR2 (Shariat *et al.* 2013a), and in a collection of 40 ser. Kentucky isolates, we found 13 CRISPR1 alleles and seven CRISPR2 alleles (Vosik *et al.* 2018).

Each isolate was attributed an Infantis CRISPR Type (ICT) identifier, based on the combination of CRISPR1 and CRISPR2 allelic profiles (Table 1). We saw a greater diversity within our collection of isolates derived from poultry (four different ICTs), than the human clinical genomes, which could be split into two CRISPR types, ICT1, and ICT4. Surprisingly, there was no diversity in the CRISPR loci among the USDA-FSIS genomes from animals, despite the wide variety in animal source (chickens, turkeys, cattle and swine). We hypothesize that the reduced CRISPR diversity might reflect the expansion of a ser. Infantis clone that has increased in circulation, particularly within human and avian populations (Gymoese *et al.* 2019). Interestingly, the ICT4 was found in 83% genomes from human clinical isolates, however, none of the poultry isolates, nor the USDA-FSIS isolates matched to this CRISPR Type.

The high conservation of the CRISPR arrays in ser. Infantis can lend themselves as ideal targets for a qPCR assay. Such an assay would have two utilities: (i) as an alternative to traditional serotyping; and (ii), to screen mixed cultures to detect background incidence of ser. Infantis that might not be detected when relying on picking one or two colonies from an agar plate. There are 31 and 26 spacer sequences in CRISPR1 and CRISPR2 respectively (Fig. 1). To identify spacers unique to ser. Infantis, we compared these sequences to a database of CRISPR spacers from 130 *Salmonella* serovars that our laboratory has collated (Thompson *et al.* 2018; Cox *et al.* 2019). The serovars represented in that database, along with information on how the database was generated, is shown in Table S1. The database includes serovars frequently associated with foodborne illness, food animals and the environment. We found that 24 spacers in CRISPR1 and 25 spacers in CRISPR2 are exclusive to ser. Infantis and not found in any other serovars (red bars in Fig. 1). The spacer sequence information is shown in Table S2. We developed two qPCR assays, one for each of the CRISPR arrays. In addition to screening the spacers against our database, we deliberately chose leader proximal spacers as targets for the qPCR assay, as leader distal spacers are commonly shared among some serovars (Fabre *et al.* 2012; Shariat *et al.* 2015). The CRISPR1 assay targets spacers 28 and 29 (spacer 30 has a low GC content, precluding it from being a good primer or probe target), and the CRISPR2 assay targets spacers 26 and 27 in their respective arrays. We further confirmed that these sequences (CR1sp28: tttcctgtaacattccgatatataattctccgc, CR1sp29: ttgattttaatggcgggcgaattgtatttaac, CR2sp26: atcaaatatcagataacccccgtcggcaaacc, and CR2sp27: tcgtacaccagcgctttaccggagtgtccgtgc) were unique to ser. Infantis by performing a BLAST search. A preliminary test demonstrated that the CRISPR2 qPCR assay was most sensitive, being able to detect as low as 10 CFU for four different serovar Infantis strains (Table 2). Subsequent experiments were therefore performed with the CRISPR2 assay. The qPCR assay was 100% specific; it did not amplify 10 other *Salmonella* serovars, nor non-*Salmonella* species (Table 3), confirming earlier bioinformatic screens when selecting which spacers to target.

Table 2 Sensitivity of Infantis qPCR assays

	CRISPR1 assay		CRISPR2 assay	
CFU	Amplification*	Ct value†	Amplification*	Ct value†
1×10^5	+	27·1	+	24·2
1×10^4	+	31·1	+	27·0
1000	+	33·0	+	30·8
100	+	37·8	+	33·6
10	+/−		+	36·1
1	−		+/−	
0·1	−		−	

+ denotes amplification, − denotes no amplification.
*,†Representative of at least three independent experiments.

Table 3 Specificity of serovar Infantis qPCR assay

Isolate	CR2 assay	Isolate source
S. infantis (040819-6)	+	This study
S. infantis (022519-13)	+	This study
S.infantis (012819-3)	+	This study
S. infantis (011819-1)	+	This study
S. Kentucky (M10019288001A)	–	Vosik *et al.* (2018)
S. kentucky (08E00076)	–	Vosik *et al.* (2018)
S. enteritidis (08E00786)	–	Shariat *et al.* (2013a)
S. typhimurium (07E00002)	–	Shariat *et al.* (2013b)
S. heidelberg (06E00416)	–	Shariat *et al.* (2013b)
S. newport (07E00097)	–	Shariat *et al.* (2013c)
S. tennessee (104296)	–	This study
S. senftenberg (118027)	–	This study
S. cerro (19-1TX)	–	This study
S. hadar (108937-7)	–	This study
Campylobacter jejuni	–	ATCC 49943
Bacillus cereus	–	ATCC 11778
Clostridium septicum	–	ATCC 12464
Escherichia coli	–	ATCC 25922
Pseudomonas aeruginosa	–	ATCC 27853
Bordetella bronchiseptica	–	ATCC 10580
Citrobacter freundii	–	ATCC 8090
Proteus vulgaris	–	ATCC 13315
Staphylococcus epidermidis	–	ATCC 12228
Staphylococcus aureus	–	ATCC 29213

There have been several qPCR assays developed to detect *Salmonella* (Malorny *et al.* 2004; Cheng *et al.* 2008; Leader *et al.* 2009; Zhang, Brown and Gonzalez-Escalona 2011; Rothrock *et al.* 2018), or individual serovars (Farrell *et al.* 2005; Arrach *et al.* 2008; Afroj *et al.* 2017; Kasturi and Drgon 2017; Souza *et al.* 2019), including some assays that target the CRISPR loci (Fabre *et al.* 2014; Bugarel *et al.* 2018). We note that the ser. Infantis qPCR assay developed in Bugarel *et al.* targets spacers 4 and 5 in the CRISPR2 locus. Spacer 4 is missing in the CRISPR arrays of the two ICT5 isolates (Fig. 1), so that particular assay would not identify these two isolates as ser. Infantis (2018).

Molecular alternatives to serotyping, such as this assay, are useful for rapid screening for a particular serovar from a collection of isolated *Salmonella* strains. However, there is an increased appreciation for the prevalence of samples that contain multiple *Salmonella* serovar. Recent work has highlighted that the incidence of mixed serovar populations in poultry is more common than previously demonstrated (Thompson *et al.* 2018; Cox *et al.* 2019). A major implication of heterogenous serovar populations includes masking of less abundant but clinically relevant serovars by serovars that are more abundant and therefore easier to detect, but that have a lower association with human illness. For example, ser. Kentucky has been the top serovar isolated from broiler carcasses during processing for several years, but in the United States is infrequently responsible for human illness (USDA-FSIS 2014; CDC 2019a). The abundance of ser. Kentucky in poultry likely masks other serovars (Thompson *et al.* 2018; Cox *et al.* 2019), and these may have phenotypes that render them more important, including higher association with human illness, or increased antibiotic resistance. Given the clinical importance of ser. Infantis and the need for increased surveillance sensitivity of this serovar, we sought to determine whether our qPCR assay was able to detect ser. Infantis in a mixed *Salmonella* culture. Using a static amount of serovar Infantis (100 CFU), we varied the amount of serovar Kentucky as a second serovar from 50 to 99·9% of the population. The Ct values for serovar Infantis were similar whether this isolate constituted the entire population (100%) or was present at a much lower relative amount (0·1%) (Table 4). This data demonstrates that the qPCR assay is able to detect ser. Infantis from mixed populations of *Salmonella.*

An ability to rapidly detect important serovars with greater sensitivity from mixed *Salmonella* populations in a directed manner is therefore a valuable tool in our diagnostic tool kit. During the 2018 outbreak investigation genetically related strains of ser. Infantis were found in over one-third of poultry processing facilities in the United States, and since the outbreak. The qPCR assay presented here is ideal to assist with the increased ser. Infantis surveillance resulting from the 2018 outbreak. It also has the added utility of being able to detect ser. Infantis when it is less abundant than other serovars, scenarios that likely exist in mixed *Salmonella* enrichment cultures.

Finally, the approach used here can be adapted to screen for other *Salmonella* serovars so long as the CRISPR spacer sequences that are targeted by the primers and probe are (i) unique to that serovar, and (ii) conserved and present in all strains of that serovar.

Materials and methods

Salmonella isolates and genomes

The *Salmonella* isolates investigated in this study are listed in Table 1 and were collected in 2019. For analysis of whole genomes available on NCBI, sequence reads from isolates sequenced by the CDC and by the USDA-FSIS were downloaded and assembled using SPAdes (Bankevich *et al.* 2012). Representative genomes for each SNP cluster on GenomeTrakr (Timme *et al.* 2018; Timme, Sanchez Leon and Allard 2019) were used from genomes submitted between January 1, 2018 and June 30, 2019. For SNP clusters with a large number of genomes, one genome per month was examined.

Table 4 qPCR assay can detect serovar Infantis in mixed cultures

% Kentucky	% Infantis	Amplification*	Ct value†
0	100	+	32·9
50	50	+	32·7
90	10	+	33·2
99	1	+	32·9
99·9	0·1	+	33·7
100	0	–	–

+ denotes amplification, – denotes no amplification.
*,†Representative of two independent experiments.

DNA isolation and PCR

Salmonella isolates were grown in 2 ml of LB in a shaking incubator overnight at 37°C. A 600 µl aliquot was used to extract total genomic DNA using the Genome Wizard kit (Promega, WI), according to the manufacturer's instructions. DNA pellets were resuspended in 200 µl of molecular grade water and stored at −20°C. CRISPR1 and CRISPR2 loci were amplified and sequenced as previously described (Shariat *et al.* 2013a). For the CRISPR1 amplicons, an additional internal primer was required to facilitate complete sequencing of the entire array, 5′-ttactcgatgcgtacctgtg-3′.

CRISPR-typing

The Sanger sequence reads for each of the CRISPR loci or the assembled genomes were examined using CRISPRFinder (Grissa *et al.* 2007) and the spacers were extracted and visualized using an Excel-based macro (Horvath *et al.* 2008; Shariat *et al.* 2013a). Different alleles were identified by the unique spacer content, as previously described (Shariat *et al.* 2015) and CRISPR Types were assigned based on the combined allelic variation of both CRISPR loci.

qPCR assays

Single *Salmonella* colonies were grown in 3 ml Luria–Bertani (LB) broth for 3 h and normalized to an optical density of 0·2 at OD_{600} using fresh LB. Serial 10-fold dilutions were made, also in LB, for use as qPCR templates. The CFU per ml was calculated retroactively. For the mixed culture experiments, isolates of ser. Infantis and Kentucky were prepared in the same manner. For ser. Infantis, 10 µl that equalled 100 CFU was mixed with 10 µl of varying amounts of ser. Kentucky as indicated in Table 4, and briefly vortexed. The serovar Kentucky and Infantis individual controls were mixed with 10 µl of LB. For the mixed culture experiments, ser. Infantis isolates 040819-6 and 012819-3, and ser. Kentucky isolates M10019288001A and 08E00076 (Vosik *et al.* 2018) were used in two independent experiments. For negative controls with other bacterial species, a single isolated colony was picked off an agar plate and used directly in the qPCR assay.

For the qPCR, 5 µl of the culture (single isolate or mixed culture) was used as a template for the qPCR, with 10 µl Primer Time master mix (IDT, IA), and 10 µmol l^{-1} of each primer and 0·5 µl of FAM-labelled probe. The conditions for the qPCR reaction were initial denaturation at 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 30 s at 55°C. The qPCR assays were performed on the qTower3 platform and analysed using qPCRsoft 4.0 software (Analytik Jena, Jena, Germany); each qPCR reaction was performed in triplicate. The primer and probe sequences are as follows (all written in 5–3′ orientation): CRISPR1 forward primer—CGTTGATTTTAATGGCGG GCGAATTG; CRISPR1 reverse primer—CGGGGAACAC GCGGAGAATTAT; CRISPR1 probe—GCGGGGATAAAC CGTTTCCTGTAACATTCCG; CRISPR2 forward primer—TCGTACACCAGCGCTTTACCG; CRISPR2 reverse primer—CGGGGAACACGGTTTGCC; CRISPR2 probe CAGCGGGGATAAACCGATCAAATATCAGATAACCC.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Serovars included in the Salmonella CRISPR spacer database.

Table S2. Serovar Infantis spacer sequence information.