1 Genomic comparison of eight closed genomes of multidrug resistant *Salmonella enterica* 2 strains isolated from broiler farms and processing plants in Trinidad and Tobago

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5 Meghan Maguire^a, Anisa S. Khan^b, Abiodun A. Adesiyun^b, Karla Georges^b, and Narjol Gonzalez-
6 Escalona^{a,#},

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9 ^aDivision of Microbiology, Office of Regulatory Science; Center for Food Safety and Applied Nutrition,
10 Food and Drug Administration, College Park, MD, USA,
11 ^b School of Veterinary Medicine, Faculty of Medical Sciences, University of the West Indies, St.
12 Augustine, Trinidad and Tobago.

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15 Running Head: Closed genomes of eight *Salmonella enterica* strains

16 Keywords: Salmonella, multi-drug resistance, complete genome, nanopore sequencing, poultry

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18 **Significance:** *Salmonella enterica* contamination of poultry is a major route of transmission to
19 humans. We have closed genomes of eight relevant multi-drug resistance (MDR) *Salmonella* strains
20 belonging to different serotypes and report multiple antimicrobial resistance (AMR) genes from
21 those strains collected in processing plants and retail markets in Trinidad and Tobago. These closed
22 genomes will be useful for future source tracking, monitoring AMR transmission, and future outbreak
23 investigations in Trinidad and Tobago and worldwide.

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25
26 [#]Address correspondence to Narjol Gonzalez-Escalona, narjol.gonzalez-escalona@fda.hhs.gov.

Abstract

2

3 *Salmonella enterica* is an important foodborne pathogen worldwide. We used long and short read
4 sequencing to close genomes of eight multidrug resistant (MDR) *Salmonella enterica* strains,
5 belonging to serovars Infantis (2), Albany, Oranienburg, I 4,[5],12:i:-, Javiana, Schwarzengrund, and
6 Kentucky from broiler chicken farms and processing plants in Trinidad and Tobago. They also
7 belonged to seven different sequence types (STs- 32, 292, 1510, 19, 24, and 96). Seven of the
8 strains demonstrated multi-drug resistance with the presence of at least three AMR genes. Three
9 isolates contained the quinolone resistance gene *qnr*_{B19} in plasmids (CFSAN103840,
10 CFSAN103854, and CFSAN103872). The extended spectrum β -lactamase genes *bla*_{CTX-M-65}
11 (CFSAN103796) and *bla*_{TEM-1} (CFSAN103852) were detected in this study. The genomes closed in
12 this study will be useful for future source tracking and outbreak investigations in Trinidad and
13 Tobago and worldwide.

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Introduction

Salmonella enterica is one of the most important foodborne pathogens in the world (González-Escalona et al. 2009). There are more than 325,000 *Salmonella* genomes in NCBI encompassing isolates from many countries (<https://www.ncbi.nlm.nih.gov/pathogens/>). Nontyphoidal *Salmonella* (NTS) is responsible for approximately 93,757,000 cases of gastroenteritis and 155,000 deaths worldwide each year. Nearly 86% are linked to foodborne transmission (Majowicz et al. 2010). Poultry, including eggs and egg products, is one of the main reservoirs of *Salmonella* and has often been implicated as the source of many outbreaks worldwide (Antunes et al. 2016; Schoeni et al. 1995; Kuehn 2010; Deng et al. 2014; Jackson et al. 2013).

Salmonella serovars Enteritidis, Newport, Typhimurium, Javiana, I 4,[5],12:i:-, and Heidelberg have been among the top five serovars associated with human illness over the last 15 years (2006-2016) in the United States ([CSL STYLE ERROR: reference with no printed form.]). *S.* serovar Infantis has shown the highest percent increase in reported cases over the same time frame (+168.5%). While, *S.* serovar Kentucky has become the most commonly detected serovar in chicken (Foley et al. 2011), but is not among the top 20 serovars causing human illness ([CSL STYLE ERROR: reference with no printed form.]).

While most *Salmonella* infections result in gastroenteritis without the need for additional medical intervention, severe, invasive infections can require antimicrobial treatment. Multidrug resistance (MDR) in *Salmonella* is closely monitored in clinical and environmental isolates around the world [National Antimicrobial Resistance Monitoring System (NARMS), European Center for Disease Control and Prevention (ECDC), and European Food Safety Authority (EFSA)]. In the United States, the FDA has issued guidance on the judicious use of antimicrobial agents in food-producing animals (<https://www.fda.gov/regulatory-information/search-fda-guidance->

[documents/cvm-gfi-209-judicious-use-medically-important-antimicrobial-drugs-food-producing-animals](#)) and individual antimicrobial agent restrictions have resulted in decreased prevalence of resistance-conferring genes (<https://www.fda.gov/media/108304/download>).

Several antimicrobial resistant (AMR) *Salmonella* strains were isolated during a *Salmonella* and AMR surveillance program of poultry production facilities in Trinidad and Tobago during 2016-2019 (Khan, et al, unpublished results). Their genomes were sequenced by short read sequencing and we selected a group of 8 strains belonging to multiple serovars and showing a diverse range of MDR, to completely close their genomes using a combination of long-read and short-read sequencing technologies. The availability of these closed genomes will be useful for future outbreak investigations and for understanding AMR acquisition and transmission in *Salmonella* within the local in Trinidad and Tobago region and worldwide.

Results and Discussion

An AMR surveillance program of poultry farms and retail outlets in Trinidad and Tobago from 2016 – 2019 produced several *Salmonella* isolates that were confirmed to be *Salmonella* by selective microbiological plating. Initial sequencing efforts identified the sequence type (ST) and serovar for each sample by *in silico* analysis. We selected eight samples from multiple serovars to completely close genomes using a hybrid assembly with Illumina MiSeq short-read and Oxford Nanopore long-read sequencing. The short-read sequencing for these eight samples was performed over three sequencing runs with an average output of 12.6 Gb and estimated average genome coverage of 50 – 150X. The multiplexed nanopore sequencing for eight samples was carried out over two sequencing runs. The output for run 1 was 7.69 Gb (2.33 million reads with a quality score of 9.48 and N50 – 11,835 bp) and for run 2 was 3.71 Gb (694 thousand reads with a quality score of 9.93 and N50 – 12,930 bp). The estimated average genome coverage was 59 - 250X (Table 1).

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2 The final closed genomes were generated by a hybrid assembly (Unicycler), which matched in
3 synteny and size to a *de novo* assembly generated using only nanopore reads by the Flye
4 assembler. *In silico* analysis confirmed the ST and serovar for each closed genome observed with
5 the preliminary analysis run using the short-read sequencing (unpublished results) (Table 1). The
6 strains represented seven different STs and serotypes (32 – Infantis, 292 – Albany, 1510 –
7 Oranienburg, 19 - 1,4,[5],12:i:- (Typhimurium), 24 – Javiana, 96 – Schwarzengrund and 152-
8 Kentucky). The genome size of these NTS strains varied from 4.6 Mb (Javiana, CFSAN103840) to
9 5.1 Mb (Oranienburg, CFSAN103872), highlighting the plasticity of NTS genomes (Table 1). A
10 cgMLST analysis showed that these strains were highly divergent but shared at least 3432 genes
11 out of a total of 4160 genes of the reference *S. Enteritidis* P125109 (NC_011294) strain used for this
12 cgMLST analysis (Figure 1A). A minimum spanning tree of the cgMLST analysis showed that they
13 differed in most loci, >78% (Figure 1B). With the two Infantis (ST32) differing in at least 136 loci,
14 which was expected since Infantis strain CFSAN103816 lacked the MDR plasmid, indicating that
15 these two strains belonged to very different clones circulating in the same country at the same time.
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17 The closed genomes were further analyzed for the presence of AMR genes using the ResFinder
18 database (Table 1) (Bortolaia et al. 2020). All strains were found to contain *mdsA*, *mdsB*, and *mdsC*
19 components of the MdsABC complex in the chromosome. MdsABC confers resistance to multiple
20 toxic compounds, including novobiocin, acriflavine, crystal violet, ethidium bromide, methylene blue,
21 rhodamine 6G, tetraphenylphosphonium bromide, benzalkonium bromide, and SDS and oxidative
22 stress-inducing compounds (diamide, H₂O₂, Paraquat), and is necessary for colonization of host
23 cells (Nishino et al. 2006; Song et al. 2015)- Seven of the strains demonstrated multi-drug
24 resistance with the presence of at least three AMR genes. Aminoglycoside, tetracycline, and
25 sulfonamide resistance genes were detected in seven strains: CFSAN103796 (*aac* (6'), *tetA*,
26 *aph*(3''), *sul1*, *aadA1*, *aac*(3)-*lva*, *aph*(4)-*la*), CFSAN103854 (*aac*(6')-*laa*), CFSAN103872 (*aac*(6')-

1 *laa*, *aph(3'')*-*lb*, *aph(6)*-*ld*, CFSAN103852 (*aac(6')*-*laa*, *aph(3'')*-*lb*, *aph(6)*-*ld*, CFSAN103816
2 (*aac(6')*-*laa*), CFSAN103862 (*aac(6')*-*laa*, *aph(3'')*-*lb*, *aph(6)*-*ld*), and CFSAN103828 (*aac(6')*-*laa*,
3 *aph(3'')*-*lb*, *aph(6)*-*ld*, *tetB*) (Table 1). In clinical *Salmonella* isolates, combined resistance to
4 ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (ACSSuT) decreased from
5 8.2% in 1996 to 3.6% in 2009 (Medalla et al. 2013), but other medically relevant antibiotics have
6 become a recent focus of study. Synthetically derived fluoroquinolones were introduced to combat
7 ACSSuT resistance. Three isolates contained the quinolone resistance gene *qnr_{B19}* in plasmids
8 (CFSAN103840, CFSAN103854, and (CFSAN103872) of different sizes. Plasmid-mediated
9 quinolone resistance was not identified in retail meat products until 2017 (Sjölund-Karlsson et al.
10 2010; Tyson et al. 2017). The presence of this small plasmids carrying *qnr_{B19}* gene is worrisome
11 since they have been identified in other countries in Latin America such as Peru, Bolivia, Colombia,
12 and Argentina and appears to be highly mobile among different microorganisms (Tran et al. 2012).

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14 β -lactams, including ampicillin and cephalosporin, are another treatment of invasive salmonellosis,
15 particularly in children. Ceftriaxone resistance increased in the US from 10% in 2002 to 38% in 2010
16 in chicken, prompting prohibition of cephalosporin by the FDA in 2012. The extended spectrum β -
17 lactamase genes *bla_{CTX-M-65}* (CFSAN103796) and *bla_{TEM-1}* (CFSAN103852) were detected in this
18 study. Brown, et al (2018) described the presence of *bla_{CTX-M-65}*-containing *Salmonella* isolates in the
19 US that were grouped in the same phylogenic clade as MDR Peruvian isolates (Brown et al. 2018),
20 illustrating the intercontinental spread of this AMR gene. We have additionally detected the
21 presence of the *mcr-9* gene in another strain isolated with this study (Maguire et al. 2021). The *mcr-*
22 *9* gene variant was described in 2019 (Carroll et al. 2019) and appears not to confer colistin
23 resistance in *Salmonella* and *E. coli* (Tyson et al. 2020).

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25 **Conclusion**

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MDR NTS presence in poultry environments remains a significant global challenge for public health. With the increasing reports of newer variants of AMR genes that confer resistance to diverse types of antibiotics, the need to enact active AMR gene surveillance studies is paramount. With these types of studies, we can not only detect the presence and spread of important AMR genes in poultry, but also identify the strains carrying these AMR genes and their location (whether in the chromosome or plasmids). Here we closed eight genomes of several MDR NTS, determine their ST, serotype, and their associated AMR gene profiles, which will be useful for future source tracking and outbreak investigations in Trinidad and Tobago and worldwide.

Materials and Methods

Bacterial strains and media. Cloacal swabs, drag swabs, whole chicken carcasses, and neck skin samples were collected from retail outlets, processing plants, and farms in Trinidad and Tobago during an AMR surveillance program from 2016 – 2019. The sample was pre-enriched in buffered peptone water (Oxoid, Ltd., Hampshire, UK) for 18 to 24 h at 37°C, then selectively enriched in tetrathionate broth (Oxoid), and thereafter incubated for 18 to 24 h at 37°C. The sample enriched in selective broth was then sub-cultured onto xylose lysine Tergitol 4 (Oxoid) and incubated aerobically at 37°C for 18 to 24 h. Suspected Salmonella colonies that displayed characteristic colonies on the selective agar plate were then purified on blood agar plates (Oxoid) and incubated at 37°C for 18 to 24 h. Pure cultures were subjected to a panel of biochemical tests that included triple sugar iron agar, lysine iron agar, urea, citrate, methyl red, sulfide-indole-motility medium, and o-nitrophenyl-b-D-galactopyranoside (Oxoid) (Andrews 1992). Biochemically confirmed isolates were then subjected to serological typing by using Salmonella polyvalent antiserum (A-I and Vi, Difco, Detroit, MI). Complete confirmation and serotyping of Salmonella isolates were performed by using the phase

reversal technique, and the results interpreted according to the Kauffman-White scheme (Grimont & Weill 2007) at the Public Health Laboratory, Ministry of Health, St. Michael, Barbados. The single isolate strains were grown overnight in tryptic soy broth (TSB) medium at 35°C.

Nucleic acid extraction. DNA was extracted using the Maxwell RSC cultured cells DNA kit with a Maxwell RSC instrument (Promega, Madison, WI) following the manufacturer's protocols for Gram-negative bacteria with additional RNase treatment. DNA concentration was determined by Qubit 4 Fluorometer (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Illumina MiSeq short-read sequencing. The short reads whole genome sequence for this strain was generated by Illumina MiSeq sequencing with the MiSeq V3 kit using 2 x 250 bp paired-end chemistry, (Illumina, San Diego, CA) according to manufacturer's instructions. The libraries were constructed using 100 ng of genomic DNA using the Illumina® DNA Prep (M) Tagmentation (Illumina), according to manufacturer's instructions. Reads were trimmed with Trimmomatic v0.36 (Bolger et al. 2014).

Oxford Nanopore long-read sequencing. The long reads for each strain were generated through GridION sequencing (Nanopore, Oxford, UK). The sequencing library was prepared using the rapid barcoding sequencing kit (SQK-RBK004). Each library was run in a FLO-MIN106 (R9.4.1) flow cell, according to the manufacturer's instructions, for 48 hours. Default parameters were used for all software unless otherwise specified. The run was base called live with default settings (MinkNOW Core v3.6.5, Bream v4.3.16, and Guppy v3.2.10). Reads < 4000 bp and quality scores of <7 were discarded for downstream analysis for an

Contig assembly and annotation. The final complete genome (chromosome and plasmid(s), when present) for each strain was obtained using a previously described pipeline (Gonzalez-Escalona et

1 al. 2018) with modifications. The final genomes were achieved by a hybrid assembly generated
 2 using both nanopore and MiSeq data with Unicycler v0.4.8 (Wick et al. 2017). A second *de novo*
 3 genome assembly was obtained with Flye v2.8 (Kolmogorov et al. 2019) using nanopore data. Both
 4 assemblies (Flye and Unicycler) for each strain were aligned with Mauve v2.4.0 (Darling et al.
 5 2004). If the two aligned assemblies agreed in synteny and size, then the Unicycler hybrid assembly
 6 was used as the final assembly (i.e. complete genome). Unicycler assembled the chromosomes and
 7 plasmids as circular closed and oriented the chromosomes to start at the *dnaA* gene. The genomes
 8 were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP,
 9 http://www.ncbi.nlm.nih.gov/genome/annotation_prok) (Tatusova et al. 2016). *In silico* analysis was
 10 used to determine the multi-locus sequence type (MLST) with the MLST website for *Salmonella*
 11 (<http://enterobase.warwick.ac.uk/species/index/senterica>), serotype using SeqSero (Zhang et al.
 12 2015) (<http://www.denglab.info/Seqero>) which infers the serovar from antigenic structure, and
 13 antimicrobial genes were found using ResFinder-4.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>)
 14 (Bortolaia et al. 2020).

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16 **Genomic data analysis.** To analyze the relationships among the eight NTS closed genomes we
 17 performed a core genome MLST (cgMLST) using Ridom SeqSphere+ v6.0.2 (Ridom GmbH,
 18 Germany) as previously described (Toro et al. 2016). The genome of *S. Enteritidis* P125109
 19 (NC_011294) was used as reference genome (4,160 genes) (Thomson et al. 2008). The *S.*
 20 *Enteritidis* genome from strain EC20121175 (CP007269.2) was used for comparison with the
 21 reference genome to establish a list of core and accessory genome genes. Genes that are repeated
 22 in more than one copy in any of the two genomes were removed from the analysis as failed genes.
 23 A task template was created that contains both core and accessory genes for this reference SE
 24 strain for any future testing. Each individual locus (core or accessory genes) from strain P125109
 25 was assigned allele number 1. The assemblies for each individual NTS closed genome in this study
 26 were queried against the task template and if the locus was found and was different from the

1 reference genome or any other queried genome already in the database, a new number was
 2 assigned to that locus and so on. After eliminating any loci that were missing from the genome of
 3 any strain used in our analyses, we performed the cgMLST analysis. These remaining loci were
 4 considered the core genome shared by the analyzed strains. We used Nei's DNA distance method
 5 (Nei et al. 1983) for calculating the matrix of genetic distance, taking only the number of
 6 same/different alleles in the core genes into consideration. A Neighbor-Joining (NJ) tree using the
 7 appropriate genetic distances was built after the cgMLST analysis.

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9 **Data deposition:** The complete genome sequences of the eight *S. enterica* strains have been
 10 deposited at GenBank under the accession listed in Table 1.

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1 **Table 1.** Metadata for the eight *S. enterica* strains isolated from chicken on poultry farms and processing plants reported in this study.

CFSAN No.	Accession No. chromosome and plasmid(s) (size bp)	GC content (%)	Hybrid assembly genome coverage (X)	Illumina SRAs (read No.)	Nanopore SRAs (read No.)	AMR genes ^a	ST ^b	Serotype ^c
CFSAN103796	CP066335 (4,727,835)	52.3	95	SRR11660184 (2,096,092)	SRR13073499 (47,009)	<i>mdsA, mdsB, mdsC,</i> <i>aac(6')-laa</i>	32	Infantis
	CP066336 (312,952)	50.3	83			<i>tetA, aph(3''), sul1,</i> <i>aadA1, aac(3)-Iva,</i> <i>aph(4)-Ia, blaCTX-M-</i> <i>65, dfrA14, qacEdelta1</i>		
CFSAN103854	CP066333 (4,944,981)	52.2	133	SRR11660185 (2,482,592)	SRR13073498 (61,306)	<i>mdsA, mdsB, mdsC,</i> <i>aac(6')-laa</i>	292	Albany
	CP066334 (3,071)	48.2	33			<i>qnrB19</i>		
CFSAN103872	CP066260 (5,053,975)	52.2	80	SRR11660187 (1,533,662)	SRR13073497 (41,126)	<i>mdsA, mdsB, mdsC,</i> <i>aac(6')-laa</i>	1510	Oranienburg
	CP066261 (49,044)	49.3	265			<i>qnrB19, aph(3'')-Ib,</i> <i>aph(6)-Id</i>		
	CP066262 (5,320)	52.4	400					
CFSAN103852	CP066328 (4,836,001)	52.2	129	SRR12995791 (2,606,746)	SRR13073496 (70,597)	<i>mdsA, mdsB, mdsC,</i> <i>aac(6')-laa</i>	19	I 4,[5],12:i:- (Typhimurium)
	CP066329 (110,489)	50.2	189			<i>aph(3'')-Ib, aph(6)-Id,</i> <i>blaTEM-1</i>		
	CP066330 (93,844)	53.1	228					
	CP066331 (33,659)	41.3	310					
	CP066332 (1,749)	57.5	1400					
CFSAN103840	CP066325 (4,554,367)	52.1	105	SRR12995802 (2,110,046)	SRR13073495 (63,239)	<i>mdsA, mdsB, mdsC,</i> <i>aac(6')-laa</i>	24	Javiana
	CP066326 (2,579)	50.5	1000			<i>qnrB19</i>		
	CP066327 (1,395)	55.2	3000					
CFSAN103816	CP066324 (4,703,182)	52.3	59	SRR12995817 (2,060,084)	SRR13073494 (53,365)	<i>mdsA, mdsB, mdsC,</i> <i>aac(6')-laa</i>	32	Infantis

CFSAN103862	CP066321 (4,847,947)	52.2	125	SRR12995807 (1,866,844)	SRR13073493 (106,256)	<i>mdsA, mdsB, mdsC, aac(6')-Iaa</i>	96	Schwarzengrund
	CP066322 (144,839)	49.0	198			<i>aph(3'')-Ib, aph(6)-Id, sitABCD</i>		
	CP066323 (4,050)	42.8	700					
CFSAN103828	CP066318 (4,767,999)	52.2	30	SRR12995770 (1,746,768)	SRR13073492 (30,574)	<i>mdsA, mdsB, mdsC, aac(6')-Iaa</i>	152	Kentucky
	CP066319 (146,829)	49.4	48			<i>sitABCD, tetB, aph(3'')- Ib, aph(6)-Id</i>		
	CP066320 (46,120)	42.8	150					

^ain silico antimicrobial genes were found using ResFinder-4.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>).

^bin silico MLST used the MLST website for Salmonella (<http://enterobase.warwick.ac.uk/species/index/senterica>).

^cin silico serotyping using SeqSero (Zhang et al. 2015) (<http://www.denglab.info/SeqSero>), a tool to infer serovar from the genes that determine antigenic structure.

1 **Figure 1.** cgMLST analysis of the eight *Salmonella* closed genomes based on 4028 shared genes. A) NJ tree showing the genetic
2 differences among these genomes based on the shared loci. B) Minimum spanning tree allowing to visualize the number of loci differences
3 between these genomes. Strains are color coded according to their ST. The ST is displayed after the name of the strain, separated by a
4 comma. Numbers by lines represent the number of loci differing between strains. The lines are not drawn to scale.

A)

B)

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