

Antibiotic Resistance and Potential Pathogenicity of an Isolate *Salmonella enterica enterica* Based on Genomic Comparison with of 103 and 2199 Strains Obtained from Contaminated Chicken Meat in Mexico

Renaud Condé¹, Pérez de la Rosa Diego², Lozano Luis³, Hernández Salgado Homero², Rocha-Martínez Karina², Rojas-Ramírez E. Edmundo⁴, Sachman-Ruiz Bernardo^{4*}

¹Instituto nacional de Salud Pública, Cuernavaca, Morelos, México

²Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, Morelos, México

³Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

⁴Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria del Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Morelos, México

Email: *sachman.bernardo@inifap.gob.mx

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Abstract

The strategies implemented to identify pathogenic strains of *Salmonella* in countries with high production and consumption when is of chicken meat [such as Mexico), successfully bring germ-free meat to the market. Two *Salmonella enterica enterica* strains obtained from Mexican chicken meat were completely sequenced. The genomic comparison with the CT18 *Salmonella* strain indicates that strains 103 and 2199 vary by 1.9%. Genome analysis of the isolated strains revealed the presence of numerous virulence genes, as well as antibiotics resistance genes in these two isolates. Their potential pathogenicity was inferred from presence of 22 (103 strains) and 19 genes (2199 strains) homologous to the one annotated in *Salmonella enterica* virulome databanks. The characterization of these strains will contribute to successful *Salmonella* monitoring in Mexico.

Keywords

Salmonellosis, Pathogenic, Genome, Antibiotic Resistance, Chicken Meat

1. Introduction

Salmonella food poisoning has raised medical concerns and propelled the need

for diagnosis and strain identification [1]. The increase of genomic data bases provides the possibility to perform entire genomic comparisons from particular isolates of variety of the principal fastidious pathogens related to food source. In particular, *Salmonella enterica enterica* food poisoning has been under scrutiny, as the pathogen can cause symptoms ranging from asymptomatic carriers to severe diarrhea [2]. Chicken meat processing has been pinpointed as a prominent contagion media. Genome analysis has been successfully used to determine the strains and variety of the contaminant microorganism. Genome data mining also permitted to relate pathogenicity genes to the disease severity [3]. The pathogenicity of a *Salmonella* strain is related to the presence of distinct genes, such as the *avrA* gene, only observed in serovars that have a potential to cause severe salmonellosis in humans [4] [5]. The vast majority of the world countries have established strict normativity regarding food supplies and particularly regarding *Salmonella sp.* presence in chicken meat [6]. Mexico chicken production for exportation (6 thousands metric tons) constitutes an important agricultural sector, employing over 1,200,000 people [7]. Therefore, identification of *Salmonella* strains infecting the chickens as well as the survey of meat edibility is crucial to economic stability. The variety of *Salmonella* strains shows distinct drug resistance patterns and virulence genes that underpin the importance of the genome typification when a new outbreak occurs. Capuano and coworkers did previously characterized 12 virulence factors (*gipA*, *gtgB*, *sopE*, *sspH1*, *sspH2*, *sodC1*, *gtgE*, *spvC*, *pefA*, *mig5*, *rck*, *srgA*) in 114 different strains of *Salmonella enterica* subsp. *enterica*. They uncovered fifty-nine unique virulence profiles, also demonstrating the importance of the combinatorial variability of these genes to determine the medical outcomes of infections. The origin of the virulence of the *Salmonella sp.* is generally attributed to presence of a subset of genes coming from prophages, though sometimes virulence factors can be transmitted by plasmids [8]. Genes related to the severity of *Salmonella* intoxication have been studied and classified as follow: *avrA* genes codes for SPI-1 effector protein inhibiting inflammation and apoptosis. The presence of this gene family would favor the *Salmonella* persistence, slowing the natural clearance of the intestinal cells of the infected patient [2]. *bcfC* genes code for an outer membrane usher protein, which is sometimes carried in the microbe genome as part of a transposable element. The pore is located in the outer membrane of the microbial pili, allowing a better bacterial protein secretion during the intestine invasion [9]. *GipA* gene family code for a short protein is involved in *Salmonella* adherence and the subsequent invasion of the macrophage, allowing their multiplication [10]. *hinS* gene is related to the flagellar stages of *Salmonella*. This gene is adjacent to the *fljB* (*H2*) gene, (phase-2 flagellin). When this gene is inverted, flagelin 2 is expressed hence allowing the microorganism movement [11].

Capuano *et al.* also determined the antibiotic sensitivity and observed a higher prevalence of plasmid borne resistance genes [8]. On the other hand, Egyptian *Salmonella* antibiotic resistance shows 73.3% resistance frequencies to chloram-

phenicol and trimethoprim-sulfamethoxazole. The same authors found that 56.7% of the bacteria were resistant to streptomycin and 53.3% to tetracycline and ampicillin. Gentamicin was overall less tolerated (30%). Cefotaxime and ceftriaxone were still efficient in 97.3% of the cases. The resistance-associated genes were *bla*TEM, *aadB*, *aadC*, *aadA1*, *aadA2*, *floR*, *tetA(A)*, *tetA(B)*, and *sul1* [12]. In order to assess the phylogenetic, the distance between two organisms, conserved genes are analyzed. In *Salmonella*, the *tufA* and *tufB* genes code for the translation elongation factors EF-Tu and are encoded by two widely separated but nearly-identical genes [13]. The analysis of their structures divergence enables the establishment of robust *Salmonella* phylogeny.

2. Methods

2.1. Samples Sources and Isolation

Sampled strains were extracted from poultry carcass sponges used for cleaning the chopping board used to carve the poultry. The procedures followed were extracted from FSIS/USDA MLG 4.09 Rev. 09. Isolation and Identification of *Salmonella* from Meat, poultry, Pasteurized Egg, and Siluriformes (Fish) Products and Carcass and Environmental Sponges Briefly, the sponges were washed in 50 mL of buffered peptone water (BPW; Oxoid, Basingstoke, England). Selective enrichment was done using a 500 µl aliquot of enriched bacteriae was re-enriched in 10 mL of Tetrathionate Broth Base, Hajna (BD Cat. 249120) and 100 µl aliquot of enriched bacteriae in 10 ml of Modified Rappaport and Vassiliadis (mRV) broth (BD Cat. 218581). Plating media were made of Brilliant green sulfa agar (BGS; contains 0.1% sodium sulfapyridine) (BD Cat. 271710) and Xylose lysine Tergitol™ 4 agar (XLT4) (BD Cat. 223420). In order to characterize the bacteria strain remaining in the differential bacteria selection step, we grew the isolates in two different screening media: Triple Sugar Iron agar (TSI) (BD Cat. 226540) and Lysine Iron Agar (BD Cat. 284920). The resulting bacterial colony was then sub-cultured in BIH media for a further 18 h at 37°C.

2.2. Biochemical Assays to Distinguish Flagellum Phases

In *Salmonella enterica*, the transition between somatic phase and flagellar phase is mediated by a post-transcriptional control mechanism. In order to determine the isolated *Salmonella* phase, we performed a serological test with polyvalent O antiserum reactive with serogroups A through I + Vi (Statens Serum Institute Cat: 44807) and SSI H (Flagellar) antisera for slide Agglutination flagellar (Statens Serum Institute Cat: 40290) antigen. We performed a biochemical test using a standardized identification system known as API20E (Biomérieux Ref 20 100). This system uses 21 miniaturized biochemical tests and a database. These tests were inoculated with a bacterial suspension and were incubated at 35°C for 22 hours. The reactions were read according to the Reading table and the Identification was obtained using the identification software (APIWEB).

2.3. DNA Extraction

DNA templates were prepared from the resulting enrichments by boiled cell lysis. Briefly, 1 ml of overnight enrichment was pelleted by centrifugation, re-suspended in 200 µl of sterile distilled water, and boiled for 10 min. The boiled cell suspensions were centrifuged, and the resulting lysate was used for PCR.

2.4. Genomic Library Construction

Genomic DNA was extracted using the Wizard genomic purification kit (Promega) and used for PCR amplification (Data not shown). DNA fragment libraries were constructed using Genomic DNA Sample Preparation Kit (Illumina catalog numb. FC-102-1001) according to the manufacturer's recommendations.

2.5. DNA Sequencing, Genome Assembly and Analysis

The sequences from the genomes libraries were obtained using 454 FLX pyrosequencing (Roche). The genome was assembled using Newbler version 2.8. Genome sequence annotation was made by RAST [<http://rast.nmpdr.org>] [5]. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NBXM000000000 and NBXN000000000. The version described in this paper is version NBXM000000000 and NBXN000000000.

2.6. Phylogenetic Species Differentiation

We used *rpoB* and *tufA* genes (Data not shown) to determine the strains closeness.

3. Results

3.1. Genomes Assembly

A total of 4,811,656 bp, 53 contigs, an N50 of 321,932 bp were obtained for the 103 strain. The 2199 strain sequencing data consist in 4,907,872 pb, 62 contigs, an N50 of 425,021. The genome size of both strains is nearly identical (a 1.9% size difference) and maintained synteny with the closest strain CT18 (**Figure 1**).

3.2. Genome Analysis

The resulting genome comprises 4748 coding proteins, 54 rRNAs, 73 tRNAs, and 52% GC content for strain 103. The 2199 strain 4874 coding proteins, 55 rRNAs, 70 tRNAs and 52 GC_content. We found 98.49% identity with *S. enterica* var. CT18 for the 103 strain and 98.35 for the 2199 strain with high synteny in both cases (**Figure 1**). CT 18 *Salmonella enterica* serovar Typhi is a pathogenic, multidrug resistant strain, hence the interest in comparing the isolated salmonella with this particular strain [14]. Interestingly, the genomic sequences of these potentially harmful strains present in 50% more ribosomal proteins coding sequences than the reference CT18 strain, which in *Salmonella*

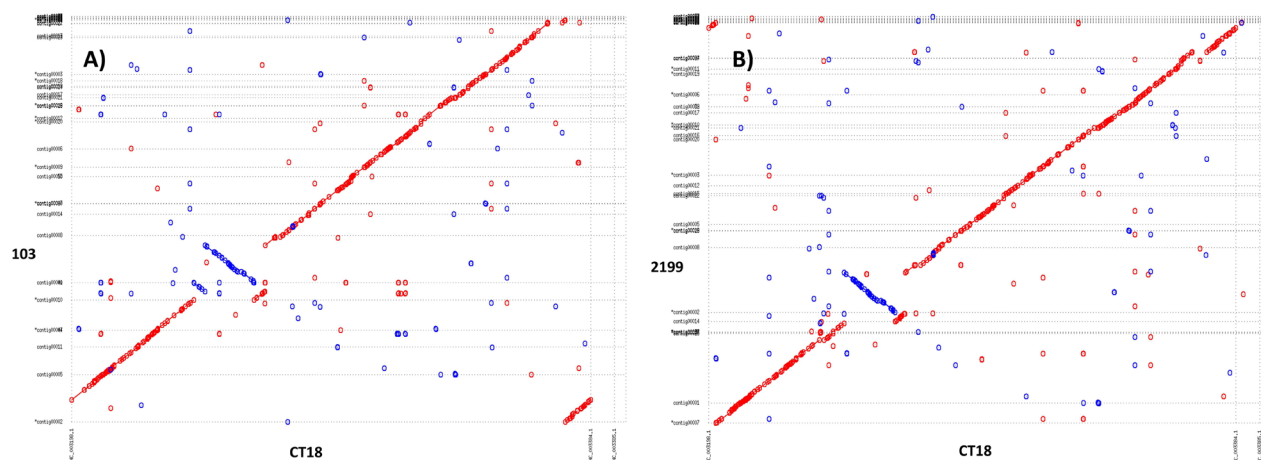


Figure 1. Shared synteny between *Salmonella enterica enterica* strains 103 (A) and 2199 (B) versus the closest reference strain CT18.

enterica is quite variable, from 67 to more than 200 elements (img.jgi.doe.gov, data not show). We could assume that this difference would be reflected in a higher protein synthesis capacity, eventually affecting their pathogenicity.

3.3. Antibiotic Resistance Genes Identified in the Sequenced *Salmonella* Strains

The resistance genes from the two *Salmonella* strains isolated from the poultry were characterized. We found that both strains shared seven resistance genes (Table 1). Common genes are the aminoglycoside 6'-N-acetyltransferase (aac and aac1) that acetylates aminoglycoside antibiotics [15], Par E providing fluoroquinolones resistance [16], TetA transporter exporting tetracyclin outside the cell [17], the QRDR Asp87 --> Gly mutation in the DNA gyrase gyrA and GyrB produces bacteria quinolone resistance [18], ParC topoisomerase mutation allowing for ciprofloxacin resistance [19] and TetA mutation allowing this enzyme to produce the oversupercoiling needed for exponential growth even in presence of tetracyclin [20]. Nevertheless, these show individual resistance genes such as cat2, a gene conferring chloramphenicol resistance [21], for the 103 strain and sul2 (sulfonamide resistance through mutation in the enzymes of the folate synthesis pathway [21]), strA and strB (streptomycin resistance gene) for the 2199 strain.

3.4. Virulence Genes Identified in the Sequenced *Salmonella* Strains

The pathogenicity of these strains is related to the presence of particular genes. The strains shared 18 virulence genes, while 4 different genes were encountered in 103 and 2 in 2199 (Table 1).

Amongst the virulence genes present only in 103 strain, GipA is a transposase allowing for the recombination of DNA of the enterobacteriae, whose presence in *E. coli* has been associated to Crohn's disease [10]. The hinS gene, correlating

Table 1. Virulence and antibiotic resistance genes present in the Salmonella isolates 103 and 2199 genomes and CT18 strain reference.

Virulence gene	103	2199	CT18	Resistance gene	103	2199	CT18
bcPC	X	X	X	aac	X	X	X
invA	X	X	X	acc1	X	X	X
inuE	X	X	X	porE	X	X	X
sopE	X	X	X	porC	X	X	X
sopB	X	X	X	syrA	X	X	X
spi4D	X	X	X	syrB	X	X	X
ssaQ	X	X	X	tetA	X	X	X
pip	X	X	X	pore2	X		X
ttrC	X	X	X	cat2	X		
misL	X	X	X	strA		X	X
sugR61	X	X		strB		X	X
phoP	X	X	X	sul2		X	X
phoQ	X	X	X	tetB			X
slyA	X	X	X	cat1			X
rmbA	X	X	X	catA			X
inoB	X	X	X	blaTEM			X
mgtC	X	X	X				
gipA	X						
hinS	X						
putTrans	X						
spoE	X		X				
rhuMg	X		X				
avrA		X					
orgA		X	X				
spiR			X				

to flagellar antigen variation [22], and a gene coding for a putative transposase are also only present in the 103 isolate. These genes could allow for antigen shifting, cloaking the *bacteriae* from the human immune system. The rhuM is a hypothetical cytoplasmic protein loosely related to STY4036 DNA Binding protein. (oncotarget 07 35169-s003]. The spoE gene, only present in 103 isolate, is a gene associated with DNA segregation and Cell cycle control in *Mycobacterium tuberculosis* [23]. Conversely, the *avrA* and *orgA* genes are only present in the 2199 isolate. The first codes for an inhibitor of the c-Jun N-terminal kinase pathway that provoke intestinal cell tight junction stabilization [24] promoting colonic tumor [25]; while the second correspond to a gene related to low oxygen environment adaptability, hence to the ability of the bacteria to strive intracellu-

larly [26].

3.5. Virulence Genes Common for Both Strains

bcfC genes codes for an outer membrane usher protein, which is sometimes carried in the microbe genome as part of a transposable element. The pore is located in the outer membrane of the microbial pili, allowing a better bacterial protein secretion during the intestine invasion [9]. invA is an internal membrane protein allowing type III secretion required for cell invasion [27]. invE is also related to secretion, allowing retention of secreted substrates within the bacterium rather than the translocation of substrates through the bacterial and/or eukaryotic membranes.

SopE is a protein required for invasion through cytoskeleton reorganization, while SopB is needed for invasion, mediating trans epithelial signaling of the polymorphonuclear leukocyte (PMNs). SopB is required for the inflammation and fluid accumulation and stimulate the recruitment of PMNs to the site of a *Salmonella* infection [28]. SPi 4 is a secretion protein related to LipC. In addition to invasion, the SPII system appears to function in programmed cell death (apoptosis) of infected cultured macrophages [29]. ssaQ *S. enterica* is a SPI2 type III secretion protein involved in cell invasion, giving an enhanced HepG2 cell invasion capacity as well as higher cytotoxicity towards macrophages [30]. spiR is a spire type nucleation factor and pipC is a protein coded in the pathogenicity island five, and is related to sigE, a stress response transcription factor. SigE activation enhances the *Salmonella* survival in the acidic environment of the phagosomal vacuole [31]. TtrC_S gene is found in the *Salmonella* LT2 serovar and is an integral membrane protein containing a quinol oxidation site required for anaerobic *Salmonella* respiration through tetraionate reduction. MisL is a *Salmonella* auto transporter outer membrane protein binding to collagen IV, thereby favoring epithelial cells invasion and colon invasion [32]. suRgi_S gene is a LT2 coded protein with ATP binding capacity that is related with bacteria virulence [33].

PhoP/PhoQ, the phoP/phoQ two-component regulatory system, controls the PrgH 45-kDa lipoprotein component of the syringe structures involved in cell secretion system [34] [35]. SlyA is a regulatory DNA binding protein, transcriptional regulator that confers resistance to oxyradicals. [36]. rmbA gi is a transcriptional regulator coded in the SPI-3 pathogenicity Island [37]. iroB gene codes for a protein with homology with bacterial glycosyl transferase and, under iron-limited growth conditions, its expression is regulated by the iron response regulator Fur [38]. MgtC virulence factor has a dual role: when bacteria localize in macrophages a part of the protein permit to withstand the acidic pH, while another part of the protein allow the *Salmonella* to thrive in low Mg (2+) environment [39].

Specific toxins related to *Salmonella* symptomatology have been hard to pinpoint, as the host susceptibility factor appears to be central for salmonello-

sis symptoms development. Comparison of the hereby reported genomes with the DBDiaSNP database failed to provide sequences directly related to diarrhea symptoms [40]. In the reported genome, we encountered SPI-4 pathogenicity island, reported elsewhere to be related to the severity of the diarrhea [41].

4. Conclusions

Rapid *Salmonella* virulent strains identification is key to the success of public health policy implementation; allowing the spread of these potentially harmful strains to the general population. We hereby present a first attempt of direct genomic analysis of potentially harmful strains in Mexico, discriminating by direct genome comparison of the presence of potential virulence genes as well as antibiotic resistance of the same. Such analysis could lead, in a foreseeable future the systematic establishment of directed PCR allowing for straightforward pathogenicity analysis. The next generation sequencing technology (454 discontinued, PacBio, Illumina, MinIon) is cheap and straightforward, easing the certification of poultry for human consumption. The health warranty of human consumption products is a crucial matter, influencing both local and international meat sale.

We conclude that strains 103 and 2199 are potentially pathogenic due to the number and characteristics of the genes of their virulomes. They also present a significant load of resistome genes, theoretically hindering infection treatment. The sequencing and typing of these *Salmonella* varieties impeached their diffusion to the public. Systematic genome sequencing will be of great help to monitor *Salmonella enterica* populations in the world.

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