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Elucidation of Antimicrobial Susceptibility Profiles and Genotyping of *Salmonella enterica* Isolates from Clinical Cases of Salmonellosis in New Mexico in 2008

Kenneth P. Smith[1], **Jeffy George**[2], **Kathleen M. Cadle**[1], **Sanath Kumar**[1], **Steven J. Aragon**[1], **Ricardo L. Hernandez**[1], **Suzanna E. Jones**[1], **Jody L. Floyd**, and **Manuel F. Varela**[1,*]

[1]Eastern New Mexico University, Department of Biology, Portales, NM, USA 88130

[2]Department of Microbiology, College of Fisheries, Mangalore - 57 5002, India

Abstract

In this study, we investigated the antimicrobial susceptibility profiles and the distribution of some well known genetic determinants of virulence in clinical isolates of *Salmonella enterica* from New Mexico. The minimum inhibitory concentrations (MICs) for various antimicrobials were determined by using the E-test strip method according to CLSI guidelines. Virulence genotyping was performed by polymerase chain reaction (PCR) using primers specific for known virulence genes of *Salmonella enterica*. Of 15 isolates belonging to 11 different serovars analyzed, one isolate of *Salmonella* Typhimurium was resistant to multiple drugs namely ampicillin, amoxicillin / clavulanic acid, chloramphenicol and tetracycline, that also harbored class 1 intergron, bla_{TEM} encoding genes for β-lactamase, chloramphenicol acetyl transferase (*cat1*), plus *floR*, *tet*(C) and *tet*(G). This strain was phage typed as DT104. PCR analysis revealed the presence of *invA*, *hilA*, *stn*, *agfA* and *spvR* virulence genes in all the isolates tested. The plasmid-borne *pefA* gene was absent in 11 isolates, while 5 isolates lacked *sopE*. One isolate belonging to serogroup E4 (*Salmonella* Sombre) was devoid of multiple virulence genes *pefA*, *iroB*, *shdA* and *sopE*. These results demonstrate that clinical *Salmonella* serotypes from New Mexico used here are predominantly sensitive to multiple antimicrobial agents, but vary in their virulence genotypes. Information on antimicrobial sensitivity and virulence genotypes will help in understanding the evolution and spread of epidemic strains of *Salmonella enterica* in the region of study.

Keywords

Salmonella enterica; minimal inhibitory concentration; PCR genotyping; antimicrobial agents; New Mexico

Introduction

The bacterium *Salmonella enterica* is a clinically-important, facultatively anaerobic rod and a member of the Enterobacteriaceae family of Gram-negative bacteria. Infectious diseases caused by a variety of *S. enterica* serotypes are widespread nationally (roughly 36,000 cases reported annually) and worldwide (over 33 million estimated annual cases), representing a serious public health concern (Kumar et al. 2008; Chuang et al. 2009). Recently, an outbreak of salmonellosis occurred in the United States involving at least 43 states and approximately 1442 patients in which serotype Saintpaul was the causative agent (CDC 2008). In particular,

*Corresponding author. Manuel.Varela@enmu.edu, Telephone: 575-562-2464; Fax: 575-562-2192.

New Mexico (NM) and Texas (TX) had relatively higher incidence rates of salmonellosis than most other states during the 2008 outbreak.

Salmonella possesses a complex mechanism comprised of a multitude of genetic factors to invade, replicate, and survive within the host. Many genes are acquired by horizontal transmission that enables the bacterium to expand the host range. These virulence genes are distributed on large genomic islands of 10-200 kb referred to as *Salmonella* pathogenicity islands (SPIs) (Marcus et al. 2000; Hensel 2004). Two well characterized pathogenicity islands SPI-1 and SPI-2 encode type III secretion systems (T3SSs). Some virulence genes not located on SPIs such as the chromosomally-encoded *stn* (*Salmonella* enterotoxin gene) and *iroB* also play important roles in the virulence of *Salmonella* (Prager et al. 1995; Bäumler et al. 1996). Further, several plasmid-borne genes such as the *spv, pefA, agfA* are essential for the attachment of *Salmonella* to intestinal epithelial cell surfaces (Feutrier et al. 1988; Bäumler et al. 1997). Environmental and clinical *Salmonella* serotypes lacking SPI-1 genes and consequently, deficient in the ability to enter cultured epithelial cells have been reported (Ginocchio et al. 1997; Hu et al. 2008).

Antimicrobial chemotherapy is indicated for food- and water-borne enteric fever and salmonellosis if *S. enterica* is the causative agent. Historically, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole were the most commonly prescribed antimicrobial agents for treatment of salmonellosis and enteric fever. Bacterial resistance to antimicrobial agents, however, effectively compromised chemotherapeutic efforts against such *S. enterica* infections (Threlfall 2002; Parry 2003). Thus, fluoroquinolones and cephalosporins were used alternatively for therapy of salmonellosis and enteric fever (Hohmann 2001). Recently, emergence has been documented of resistance determinants to antimicrobial agents belonging to both classes of the fluoroquinolones and the cephalosporins in clinical isolates of *S. enterica*, thus further confounding chemotherapeutic efforts (Kumar et al. 2008). For instance, multi-drug efflux pumps confer resistance to fluoroquinolones in *S. enterica*, and extended-spectrum β-lactamases confer resistance to the cephalosporins (Parry and Threlfall 2008).

In order to reestablish clinical efficacy, it would be important to determine the extent to which specific antimicrobial agents are effective against *S. enterica* which are implicated in outbreaks. The objective of this study was to determine the antimicrobial agent susceptibility profile for *S. enterica* clinical isolates that were responsible for cases of salmonellosis in NM. Such antimicrobial susceptibility data is necessary for ultimately circumventing resistance determinants and in establishing practices which prevent conditions that foster emergence of antimicrobial resistance mechanisms. We further PCR characterized the *Salmonella* clinical isolates for well known virulence genes. Since different serotypes were implicated in human infections in NM, genotyping of these isolates would facilitate comparison of the relative pathogenicity of *Salmonella* serotypes and a better understanding of their epidemiology.

Materials and methods

Isolation, identification, and maintenance of *Salmonella* cultures

Fifteen clinical isolates of *Salmonella enterica* were obtained from the Biological Sciences Bureau of the New Mexico State Laboratory Division (Albuquerque, NM, USA). These isolates represented a random sampling of salmonellosis cases occurring in NM in 2008. Immediately upon arrival in the laboratory, each strain was sub-cultured on Luria Bertani (LB) agar slants for routine analysis. The isolates were archived at -80°C in LB broth containing 15% glycerol. Serotyping of *Salmonella* isolates was done by the New Mexico State Laboratory in accordance with the scheme of Brenner and McWhorter-Murlin (1998). Biochemical tests were performed using BBL Crystal Identification panels according to the manufacturer's specifications (Becton Dickinson, Sparks, MD).

Antimicrobial susceptibility analysis

The minimal inhibitory concentrations (MIC) for the fifteen isolates were determined for a panel of fourteen antibiotics using E-test strips (bioMérieux, Hazelwood, MO) according to the manufacturer's instructions. The antibiotics tested included cefotaxime (CT), gatifloxacin (GA), ofloxacin (OF), imipenem (IP), levofloxacin (LE), amikacin (AK), ampicillin (AM), cefalotin (CE), trimethoprim / sulfamethoxazole (TS), ciprofloxacin (CI), gentamicin (GM), chloramphenicol (CL), amoxicillin / clavulanic acid (XL), and tetracycline (TC). Threshold MICs representing clinical resistance were determined according to CLSI standards (CLSI 2006).

Molecular analysis

The primers used for PCR detection of virulence genes *invA, hilA, iroB, agfA, pefA, vsdA, sopE, spvR* and *stn* of *Salmonella* are listed in the Table 1. Pure genomic DNA from the isolates was extracted following the protocol of Ausubel et al. (1995). All PCR amplifications were carried out in 30 μl reaction volumes consisting of a 10×buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% Nonidet P40), 30 pico moles each of forward and reverse primers, 200 μM concentrations of each of the four dNTPs, one unit of *Taq* polymerase (Fermentas, MD, USA) and 500 ng of template DNA (negative controls included no template or appropriate strain lacking target genes). PCR amplifications were performed using primers and the protocols previously described for *invA* (Rahn *et al*. 1992), and *pefA, shdA, sopE* and *spvR* (Pasmans et al. 2003). For detection of *hilA, iroB, agfA* and *stn* genes using primers designed in this study, the PCR cycling conditions consisted of an initiation denaturation of 94°C for 5 min, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. The products of PCR were separated on a 2% agarose gel, stained with ethidium bromide (0.5 μg/mL) and photographed using Kodak Gel Logic 1500 (Carestream Molecular Imaging, New Haven, CT). *Salmonella* Typhimurium (ATCC14028) was used in *invA, hilA, spvR, iroB, agfA, shdA* and *stn* PCR assays, while *S*. serotype Typhi (Presque Isle Cultures, Erie, PA) was used in *pefA* and *sopE* PCR as reference strains.

PCR analysis of antibiotic resistance genes encoding TEM type β-lactamase *bla*TEM, chloramphenicol acetyl transferase gene *catA1*, tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(G), and the gene responsible for streptomycin resistance *strA* was performed as previously described by (Aarestrup et al. 2003). PCR analysis for the florfenicol / chloramphenicol resistance gene *floR* was performed using the method of Khan et al (2000). The presence of class 1 integrons was determined by the Int-PCR assay (Rao et al. 2008). Phage typing for the DT104 phage was performed on this strain by PCR using the method of Pritchett et al (2000).

Results

Biochemical and serological characterization of *Salmonella* spp

All isolates used in this study exhibited biochemical phenotypes typical of *Salmonella enterica* that included positive methyl red-Voges Proskauer test, utilization of citrate, sorbitol, melibiose, mannose, mannitol, galactose, decarboxylation of lysine; negative for the production of indole, utilization of sucrose, and urea. All isolates exhibited alkaline slant, acid deep phenotype on triple sugar iron agar (TSIA) (Data not shown). The isolates belonged to 11 serotypes, namely Saintpaul, Typhimurium, Enteritidis, Newport, Javiana, Oranienberg, Infantis, Braenderup, Bovismorbificans, Poona, and Sombre (Table 2).

Antimicrobial susceptibility analysis

One isolate NMT-6 (*S*. Typhimurium) was resistant to ampicillin (MIC > 256 μg), chloramphenicol (MIC > 256 μg), tetracycline (MIC = 53.3 μg) and amoxicillin / clavulanic acid (MIC = 16 μg) (Table 3). All other isolates were sensitive to cefotaxime, gatifloxacin, ofloxacin, imipenem, levofloxacin, amikacin, ampicillin, cefalotin, trimethoprim / sulfamethoxazole, ciprofloxacin, gentamicin, chloramphenicol, amoxicillin / clavulanic acid, and tetracycline.

PCR detection of *Salmonella* virulence genes

All isolates of *S. enterica* used in our study were positive for *invA*, *hilA*, *spvR*, *afgA* and *stn* irrespective of serotypes (Table 2). Except *S*. Sombre (NMSO-15), all isolates were also positive for *iroB*. Six isolates, namely NMT-6 (*S*. Typhimurium), NMO-9 (*S*. Oranienberg), NMI-11 (*S*. Infantis), NMB-12 (*S*. Braenderup), NMBO-13 (*S*. Bovismorbificans) and *S*. Sombre (NMSO-15) were negative for the *sopE* sequence. Only three isolates, NMT-6 (*S*. Typhimurium), NME-7 (*S*. Enteritidis), and NMBO-13 (*S*. Bovismorbificans) were positive for plasmid-borne *pefA* by PCR. Twelve isolates harboured *shdA* while three isolates NMT-6 (*S*. Typhimurium), NMB-12 (*S*. Braenderup) and NMSO-15 (*S*. Sombre) lacked *shdA*. All *S. Saintpaul* isolates displayed uniform virulence genotypes, being negative for only the *pefA* sequence. Interestingly, *S*. Enteritidis (NME-7) harboured all virulence genes examined in this study (Table 2).

Detection of antibiotic resistance genes

The multiply-resistant isolate of *S*. Typhimurium (NMT-6) resistant to ampicillin, chloramphenicol, and tetracycline harbored the genes known to be conferring resistance to these antibiotics (Chopra and Roberts 2001). The isolate was PCR positive for bla_{TEM}, chloramphenicol acetyl transferase gene *cat1*, florfenicol / chloramphenicol resistance gene *floR*, tetracycline resistance genes *tet*(C), and *tet*(G), class 1 integrons, and the DT104 phage type.

Discussion

Here we found that clinical *Salmonella enterica* from NM belonging to 11 different serotypes were largely susceptible to clinically relevant antimicrobial agents, such as cephalosporins (cefalotin and cefotaxime), fluoroquinolones (ciprofloxacin, levofloxacin, ofloxacin, and gatifloxacin), trimethoprim / sulfamethoxazole, and imipenem (Table 3). Apart from these antimicrobial agents, all of the isolates were sensitive to amikacin and gentamicin. On the other hand, one isolate NMT-6 (*S*. Typhimurium) showed clinical levels of resistance to ampicillin, chloramphenicol, tetracycline, and amoxicillin / clavulanic acid, but was susceptible to all other antibiotics used. Five representative Saintpaul NM isolates used in this study correspond to the national (USA) outbreak of salmonellosis during the summer of 2008; these isolates were susceptible to all antimicrobial agents used in this study. The incidence of multi-resistant *S*. Typhimurium has been documented worldwide (Threlfall 2002). In comparison, the epidemics of *S*. Saintpaul is of recent origin and so far, multiple drug resistance in this serotype has not been observed among clinical isolates in the USA.

Although NMT-6 was not tested for the previously described resistance profile (Threlfall 2000), the presence of the *floR*, bla^{TEM}, *tet*(G), *spvC*, *invA*, and *int* genes, the DT104 phage type, and two integrons of 1000 bp and 1200 bp, indicate that *S*. Typhimurium (NMT-6) is a potential DT104 strain, a multidrug resistant pathogen that is relevant on an international level (Khan et al. 2000, Pritchett et al. 2000, Threlfall 2000).

In the present study, *Salmonella* strains were analyzed for the presence of 9 important virulence genes known to be essential for colonization of the human intestine and establishing infections, namely, *invA, hilA, iroB, agfA, pefA, shdA, sopE, spvR* and *stn*. The products of these genes are known to be essential during various stages of infection of the host (Marcus et al. 2000). All isolates of *Salmonella* in our study were positive for *invA, hilA, spvR, afgA* and *stn*, while *iroB, a* Fur-regulated gene was detected in all serotypes except *S*. Sombre. The significance of this latter finding is unclear. Our results further emphasize the utility of targeting virulence genes such as *invA, hilA* and *afgA* for detection of *Salmonella* (Prager et al. 1995; Malorny et al. 2003; Pathmanathan et al. 2003).

The *spv* operon (*Salmonella* plasmid virulence) is present on large plasmids of 50-90 kb found in few serotypes of *Salmonella* subspecies 1. This 7.8 kb *spv* operon, comprised of genes *spvRABCD*, is considered important for the survival and multiplication of *Salmonella* in the reticulo-endothelial system (Gulig et al. 1993; Marcus et al. 2000). *agfA* detected in all isolates encodes a thin aggregative fimbrin (AgfA) essential for colonization of host intestinal epithelial cells by attachment to glycoprotein or glycolipid receptors on epithelial cell surfaces. Previous studies have also reported that *agfA* are commonly present in human and animal isolates of *Salmonella* (Doran et al. 1993; Pasmans et al. 2005).

Several effector proteins encoded on SPI-1 are known to be essential during different stages of *Salmonella* infection. Of these, SopE encoded by *sopE* on SPI-1 is known to facilitate the entry of *Salmonella* by activation of Rho GTPases leading to cytoskeletal rearrangements of host cells, and cytokine production and the presence of this gene have been associated with the epidemic strains of *S.* Typhimurium (Mirold et al. 1999). All 5 *S*. Saintpaul isolates sourced from a recent nation-wide outbreak (CDC 2008) were positive for *sopE*, together with NME-7, NMN-8, NMJ-10, NMP-14. Six other serotypes were negative for the *sopE* sequence (Table 2).

Other genes tested in this study such as *pefA* and *spvR* are found only on plasmids (Rotger and Casadesúus 1999). The *pefA* gene encoding PefA (plasmid encoded fimbriae) plays an important role in attachment of *Salmonella* to intestinal epithelial cell surfaces (Feutrier et al. 1988; Bäumler et al. 1997). *spvR* and *pefA* may be present on the same or different plasmids. Some studies have reported *Salmonella* serotypes lacking *pefA* despite possessing virulence plasmids (Rychlik 1998). In our study, though all the isolates were positive for *spvR*, only 3 isolates, NMT-6 (*S*. Typhimurium), NME-7 (*S*. Enteritidis), and NMBO-13 (*S*. Bovismorbicans) were positive for *pefA* by PCR, suggesting that the plasmid harboring *spv* lacked *pefA* in isolates negative for *pefA* by PCR. It has been shown that some plasmids of *Salmonella* are self transmissible, implying that the presence of virulence plasmids in our strains could contribute to the spread of virulence genes and enhance the pathogenicity of other strains (Ahmer et al 1999). Thirteen isolates were positive for *shdA* encoding an outer membrane fibronectin-binding protein and located on a virulence island present only in members of *S. enterica* subspecies I (Kingsley et al. 2002). The product of this gene, ShdA, is responsible for the prolonged shedding of the subspecies I serotypes with the feces. Three isolates NMT-6 (*S*. Typhimurium), NMB-12 (*S*. Branederup) and NMSO-15 (*S.* Sombre) lacked *shdA*.

Overall, our PCR genotyping results suggest less prevalence of plasmid-borne *pefA* genes in addition to the absence of *sopE* sequences in the NM isolates of *Salmonella enterica*. NMSO-15 (*S*. Sombre) was PCR negative for four virulence genes *pefA, iroB, shdA* and *sopE*, while NMB-12 (*S*. Braenderup) lacked *pefA, shdA* and *sopE*. In contrast, *S.* Enteritidis (NME-7) possessed all virulence genes examined in our study.

In conclusion, though a multi-drug resistant and potential DT104 *S*. Typhimurium was identified among clinical isolates of *Salmonella* from NM, other serotypes including *S*. Saintpaul from a nation-wide outbreak are sensitive to important antimicrobials. Despite the demonstrated importance of various virulence genes in the pathogenicity of *Salmonella*, isolation of clinical strains deficient in one or more virulence genes indicates that several, yet to be determined, virulence factors may play a role in its enteropathogenicity. The genotyping data on clinical *Salmonella* serotypes including the outbreak strains will be valuable in future studies on the prevalence of specific genotypes and emergence of new genotypes of the same serotypes in the region of this study.

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Table 1

Primers used in this study for virulence genotyping

Target gene	Gene product/ function	Primer (5'-3')	Product size (bp)
invA	Invasion	gtgaaattatcgccacgttcgggcaa tcatcgcaccgtcaaaggaacc	284
iroB	Iron acquisition	ggagtcgttggaccactgat cggaagatgcaggatgattt	404
agfA	Fimbriae	ggattccacgttgagcattt gttgttgccaaaaccaacct	312
hilA	Global regulator	ttaacatgtcgccaaacagc gcaaactcccgacgatgtat	216
pefA	Plasmid-encoded fimbriae	acacgctgccaatgaagtga actgcgaaagatgccacaga	450
shdA	Fibronectin-binding	ctgacgttaagcggcgataa cgtcaacgtctgtcagtgta	625
sopE	Effector protein	cagacccgtgaagctatact aattgctgtggagtcggcat	380
spvR	Virulence plasmid	caggttccttcagtatcgca tttggccggaaatggtcagt	310
stn	Enterotoxin	gaagcagcgcctgtaaaatc gctgactcaggtgctgttga	405

Table 2

Results of PCR genotyping of *Salmonella* isolates for virulence genes

		Virulence genes detected by PCR								
Isolate	**Serotype**	***invA***	***hilA***	***stn***	***iroB***	***agf A***	***spvR***	***shdA***	***sopE***	***pefA***
ATCC 14028	*S.* Typhimurium	+	+	+	+	+	+	+	-	-
NMS-1	*S.* Saintpaul	+	+	+	+	+	+	+	+	-
NMS-2	*S.* Saintpau	+	+	+	+	+	+	+	+	-
NMS-3	*S.* Saintpaul	+	+	+	+	+	+	+	+	-
NMS-4	*S.* Saintpaul	+	+	+	+	+	+	+	+	-
NMS-5	*S.* Saintpaul	+	+	+	+	+	+	+	+	-
NMT-6	*S.* Typhimurium	+	+	+	+	+	+	-	-	+
NME-7	*S.* Enteritidis	+	+	+	+	+	+	+	+	+
NMN-8	*S.* Newport	+	+	+	+	+	+	+	+	-
NMO-9	*S.* Orainienberg	+	+	+	+	+	+	+	-	-
NMJ-10	*S.* Javiana	+	+	+	+	+	+	+	+	-
NMI-11	*S.* Infantis	+	+	+	+	+	+	+	-	-
NMB-12	*S.* Braenderup	+	+	+	+	+	+	-	-	-
NMBO-13	*S.* Bovismorbificans	+	+	+	+	+	+	+	-	+
NMP-14	*S.* Poona	+	+	+	+	+	+	+	+	-
NMSO-15	*S.* Sombre	+	+	+	-	+	+	-	-	-

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Table 3

Minimum inhibitory concentrations (MICs) of antimicrobial agents to *Salmonella* tested in this study

	Antimicrobial Agents MIC (μg)													
Isolate	**CT**	**GA**	**OF**	**IP**	**LE**	**AK**	**AM**	**CE**	**TS**	**CI**	**GM**	**CL**	**XL**	**TC**
NMS-1	0.094	0.032	0.125	0.25	0.064	2	1.83	3	0.07	0.17	0.37	5.3	1.16	4
NMS-2	0.064	0.032	0.125	0.23	0.047	2	2.16	2.33	0.07	0.23	0.37	5.33	1.75	4
NMS-3	0.064	0.023	0.125	0.19	0.047	1.5	2	24	0.11	0.047	0.75	4	1	4
NMS-4	0.094	0.039	0.125	0.19	0.047	2	1.5	3	0.09	0.032	0.5	4	1.5	4
NMS-5	0.047	0.032	0.094	0.125	0.064	2.25	1.83	3	0.06	0.016	0.37	3	1.5	3
NMT-6	0.094	0.032	0.125	0.125	0.064	2	R	4	0.2	0.032	1	R	16	53.3
NME-7	0.032	0.032	0.125	0.125	0.064	1.5	2	16	0.11	0.04	0.47	3	1	2
NMN-8	0.094	0.032	0.125	0.19	0.064	2	1.5	3	0.1	0.016	0.31	4	1	2
NMO-9	0.094	0.023	0.125	0.19	0.064	1.5	2	4	0.09	0.017	0.62	6	0.87	5.5
NMJ-10	0.047	0.023	0.094	0.125	0.064	2	2.5	3	0.08	0.023	0.62	5	1.5	4.5
NMI-11	0.094	0.016	0.125	0.125	0.032	1.5	1	3	0.06	0.047	0.62	4	0.66	1.5
NMB-12	0.047	0.023	0.064	0.19	0.064	1.5	2	6	0.13	0.016	1	6	1	8
NMBO-13	0.064	0.023	0.125	0.125	0.032	1.5	1	6.75	0.06	0.027	0.56	4	1.5	3
NMP-14	0.064	0.023	0.125	0.19	0.032	4	1.5	2	0.05	0.013	0.94	2	1.5	3
NMSO-15	0.032	0.016	0.064	0.125	0.032	1	1.875	2	0.02	0.014	0.845	2.2	1	2

R: MIC>256

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