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Short Communication

Salmonella Panama: Genetic Diversity of the Isolates Collected from Human and Non-human Sources

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Abstract

Introduction: *Salmonella enterica* serovar Panama belongs to the D1 serogroup and is frequently associated with nontyphoidal salmonellosis in humans. This study aimed to characterize isolates collected from Northeast Brazil by phenotypic and molecular methods. **Methods**: Forty four *S*. Panama strains were examined for antimicrobial susceptibility, virulence genes, and pulsed field gel electrophoresis (PFGE) types. **Results**: All strains were susceptible to antibiotics (except for streptomycin), presented classical virulence factors, and could be clustered into four groups and 18 pulsotypes. **Conclusions**: This work calls for continuous surveillance for the emergence of antibiotic resistance and new clones in a geographical area.

Keywords: *Salmonella* Panama. Antimicrobial susceptibility. Virulence genes. PFGE typing. Northeast Brazil.

Nontyphoidal *Salmonella* serotypes are some of the most common pathogens involved in food-borne self-limiting gastrointestinal illness worldwide that most commonly do not require antimicrobial therapy. However, these pathogens can also lead to life-threatening systemic infections such as sepsis, meningitis, arthritis, and osteomyelitis, which require specific chemotherapy[1]. Studies have suggested that previous occurrence of gastroenteritis by *Salmonella* group D in children is a risk factor for bacteremia[2]. *S. enterica* serovar Panama belongs to group D1 and has been isolated from humans, foods, domestic and wild animals, and environmental sources. In humans, *S*. Panama usually causes gastroenteritis, but tends to cause invasive illnesses in some hosts[1,2].

The ability of *Salmonella* spp. to cause disease can be attributed to an array of virulence genes located in the chromosomes or in large virulence-associated plasmids[3]. Several factors related to the virulence of *Salmonella* spp. have been described, including the presence of fimbriae and flagella, mobility, ability to invade and replicate in epithelial cells, resistance to complement action, and the production of enterotoxin, cytotoxin, and endotoxin. Some of these classic virulence factors are encoded by *invA*, *fimA*, *agfA*, *sfbA, phoP/Q*, *slyA*, *spvC*, and *fliC* genes. Many of these virulence genes of *Salmonella* spp. are found on a "Salmonella pathogenicity island" (SPI), which are genetic elements on the chromosomes acquired by horizontal gene transfer. The acquisition of SPIs is considered an important virulence trait for different *Salmonella* serotypes, allowing colonization at specific regions and increasing the capacity to pass through host barriers[4].

Few studies have examined the virulence genes in *S*. Panama[5,6]. A study with strains isolated from animals in Spain demonstrated that some virulence profiles were serovar-specific and that *S*. Panama harbored all the virulence genes tested such as *invE/A*, *phoP/Q*, *stn*, *iroB*, *slyA*, *hin/H2*, and *agfA*[6]. Another study, also carried out in Spain, with isolates from different sources, showed that all the strains of *S*. Panama examined were negative for *spvC*, which is a plasmid-located virulence

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gene, but were positive for *invA, phoP, stn,* and *slyA*, which are chromosomally located genes[5].

The worldwide emergence of multidrug-resistant *Salmonella* strains has been described[1,7,8] and these strains are usually isolated from patients with bacteremia and other complications[1]. *S*. Panama isolates from various origins exhibit antimicrobial resistance, and multidrug-resistant strains are common. In addition, *S*. Panama infections frequently recur[8,9,10].

The application of pulsedfield gel electrophoresis (PFGE) for assessing genetic diversity of *Salmonella* isolates and the clonal transmission of isolates has been reported in both the food and livestock industries. In Brazil, some studies have demonstrated the occurrence of *S*. Panama mainly in water, poultry and swine meat, in foods such as salami, and in the environment, however, there is little information available on the virulence and genetic diversity of *S*. Panama strains[9,11].

The aim of the present study was to characterize *S*. Panama isolates, using phenotypic and molecular methods. A total of 44 *S*. Panama isolates obtained from human, animal, and food sources were used in this study (**Table 1**). These strains were isolated from the Northeastern region of Brazil between 2001 and 2008 and stored at the Enterobacteria Collection of the National Reference Laboratory of Enteric Diseases (NRLED), Oswaldo Cruz Institute, Rio de Janeiro, Brazil. At the time of this study, all the *S*. Panama strains present in the collection were analyzed. The phenotypic identification of the isolates was confirmed by standard biochemical laboratory methods. Serological tests were performed by slide agglutination[12]. Susceptibility to nalidixic acid, ampicillin, ciprofloxacin, chloramphenicol, spectinomycin, streptomycin, nitrofurantoin, tetracycline, and sulfonamides was determined by the disk-diffusion method[13]. Virulence genes were detected by PCR using the primers listed in **Table 2**. Assays were performed in a final volume of 25 µl consisting 2.5 µl template *Salmonella* DNA that was added to 17.5 µl of ultrapure water (MilliQ), 2.5 µl of 10X PCR buffer (Promega Co., Madison, WI, USA), 0.75 µl of $MgCl_2$ (50 mM) [Promega Co., Madison, WI, USA], 0.5 µl of dNTP mix 10 mM (dATP, dGTP, dCTP, and dTTP) [Biotools B&M Labs, S.A.], 0.5µl of each primer (5 pmol/µl), and 0.25 µl of 1U/µl Taq DNA polymerase (Biotools B&M Labs, S.A.). Amplifications were carried out using the following program: a hot start cycle of 94°C for 2min, followed by 30 cycles of 94°C for30 s, 56°C for 30 s, and a final extension step of 72°C for 2min. Aliquots of 10 µl of the amplification products were analyzed by electrophoresis on 2% agarose (Invitrogen) gels. Gels were stained with ethidium bromide (0.5 µg/ml) and visualized with UV light using a DNR Bio-Imaging Systems (BioAmerica, Miami, Florida, USA) with a Gel Capture software version 5.0.

TABLE1: *Salmonella* Panama isolates included in this study.

No./Year[a]	Surce	State[b]	No./Year[a]	Source	State[b]
231/2001	Food	PE	1828/2006	Chicken carcass	PE
237/2001	Food	PE	15626/2006	Chicken carcass	PE
2444/2001	Human feces	PE	15637/2006	Chicken carcass	PE
2456/2001	Human feces	PE	**13506/2007**	Unspecified animal	PE
2457/2001	Human feces	PE	**6118/2007**	Chicken carcass	PE
2458/2001	Human feces	PE	**6119/2007**	Chicken carcass	PE
2460/2001	Human feces	PE	**9164/2007**	Chicken sausage	PE
2462/2001	Human feces	PE	**9165/2007**	Chicken sausage	PE
2466/2001	Human feces	PE	**9166/2007**	Chicken sausage	PE
7205/2002	Rectal swab of lizard[c]	BA	**9167/2007**	Chicken sausage	PE
7206/2002	Rectal swab of lizard[c]	BA	9168/2007	Ground meat	PE
7207/2002	Rectal swab of lizard[c]	BA	9169/2007	Ground meat	PE
9066/2002	Pork sausage	PE	**9170/2007**	Ground meat	PE
3719/2002	Human feces	BA	**9171/2007**	Ground meat	PE
5900/2003	Chicken carcasses	PE	**9854/2007**	Pork sausage	PE
5902/2003	Chicken carcasses	PE	**9431/2007**	Pasteurized milk	RN
5907/2003	Chicken carcasses	PE	**9433/2007**	Pasteurized milk	RN
4813/2003	Human feces	SE	**1900/2007**	Human feces	CE
10037/2003	Estuarine water	SE	**1901/2007**	Human blood	CE
10038/2003	Estuarine water	SE	**7866/2007**	Human feces	PE
1326/2004	Chicken carcass	SE	**620/2007**	Human feces	RN
6903/2005	Food	CE	**2625/2008**	Human-CSF[d] (four months old)	CE

[a] Original number NRLEB-Bacterial Collection, Oswaldo Cruz Institute, RJ, Brazil/ year of isolation [b]States of the northeast region of Brazil:PE, Pernambuco; BA, Bahia; SE, Sergipe; RN, Rio Grande do Norte; CE, Ceará [c]*Tupinambis merianae.* [d]CSF (cerebrospinal fluid).

TABLE 2: Primers used in the present study for detecting virulence genes.

Target genes factor	Virulence	[Name][a] / Sequence (5′ - 3′)	Amplicon size (bp)	Reference	Accession No.
sfbA	Fimbrial operons	[*sfbA*-F/R] ACC GAC CGC TGG GAT GGG/ TAA TCG TGA TGA GCC CCA ACG	146	This study	AF102556
agfA	Aggregative fimbriae	[*agfA*-F/R] CCG GCC CGG ACT CAA CG/ CCG TAT TGG CCG ACA GTA A	241	This study	U43280
invA	Invasion	[*invA*-F/R] ACA GTG CTC GTT TAC GAC CTG AAT/ AGA CGA CTG GTA CTG ATC GAT AAT	244	Chiu & Ou 1996	--
phoP/Q	Transcriptional regulator	[*phoP/Q*-F/R] ATG CAA AGC CCG ACC ATG ACG/ GTA TCG ACC ACC ACG ATG GTT	299	Way et al. 1993	--
fimA	Fimbrial operons	[*fimA*-F/R] AGC GTG AGT GGC GGT ACT A/ GCA GCG TAT TGG TGC CTT C	385	This study	S76043
fliC	Phase-1 flagellin-encoding	[*fliC*-F/R] CGT AAC GCT AAC GAC GGC/ GCA TGA GTG TCG TAA CCCG	404	This study	M84974
spvC	Virulence plasmid	[*spvC*-F/R] ACT CCT TGC ACA ACC AAA TGC GGA/ TGT CTT CTG CAT TTC GCC ACC ATCA	571	Chiu & Ou 1996	--
slyA	Transcriptional regulator	[*slyA*-F/R] GCC AAA ACT GAA GCT ACA GGT G/ CGG CAG GTC AGC GTG TCG TGC	700	Soto et al. 2000	--

PFGE was performed according to the standardized Salmgene and PulseNet protocol[14]. *S. enterica* serotype Braenderup H9812 was used as the molecular size marker in the PFGE experiment. DNA patterns were analyzed using BioNumerics software (V 4.1, Applied Maths, Kortrijk, Belgium). Algorithms available within the program were used to compare patterns. Dendrograms were produced using the Dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA), with a 1% tolerance limit and 1% optimization. Isolates with more than 80% similarity were clustered as highly genetically related and were placed into dendrogram branches numbered 1 to 14. Genetically indistinguishable isolates (similarities less than 80%) were assigned a capital letter: A, B, C, or D.

The characteristics of the 44 *S.* Panama strains included in the study are presented in **Table 1**. Among the 14 human strains, 12 were obtained from the feces of patients with gastroenteritis and two strains were isolated from patients with an invasive infection, both living in Ceará state; strain 1901was isolated from the blood of an adult woman in 2007 and strain 2625 was isolated from the cerebrospinal fluid (CSF) of a 4-month-old child in 2008. The other 30 strains were isolated from food (n=24), animals (n=4), and water (n=2).

All strains were found to be susceptible to ampicillin, ciprofloxacin, chloramphenicol, nalidixic acid, sulfonamides, tetracycline, and spectinomycin, but only 6.8% of the strains were susceptible to streptomycin. Similar resistance rates to streptomycin have been identified in some other studies[15,16]. In developed countries, antimicrobial drug resistance in nontyphoidal salmonella is commonly related to the use of antimicrobial drugs in animals used as sources of food, but in contrast, in developing countries, the occurrence of resistance in both nontyphoidal and typhoidal *Salmonella* spp. has been associated with the use of antimicrobials in human medicine. In addition, the occurrence of resistant *Salmonella* spp. isolates harboring resistance genes in plasmids and integrons can lead to the dissemination of these genes at the inter- or intra-species level. This possibility justifies the need for regular epidemiological surveillance to detect the emergence of antimicrobial-resistant *Salmonella* strains, which are of great concern worldwide.

The detection of virulence-related genes showed that all 44 isolates were positive for *invA* (invasion), *fimA* and *agfA* (adherence), and *sfbA* (component of the iron transport system) genes and negative for *spvC* (motility) and *fliC* (adherence and invasion) genes. Only one isolate (231/2001) was negative for *phoP/Q* (intra-macrophage survival),while *slyA* (a virulence-associated transcriptional regulator) was detected in 12 isolates obtained from food (7), estuarine water (2), and rectal swab from a lizard (3). *Salmonella* infections show multifactorial pathogenesis and that multiple virulence genes may be involved. Almost all the isolates analyzed in this study showed the presence of *invA, fimA, agfA, sfbA,* and *phoP/Q* genes, but not the *spvC* and *fliC* genes. These results are similar to those

reported in other studies[6]. Only 40% of our isolates contained the *slyA* gene; this result differs from results of other studies that have detected this gene in the majority of *S.* Panama isolates. The presence of these virulence genes could play an important role and contribute to the capacity of this serotype to cause disease, including invasive illnesses such as sepsis and meningitis. Experiments carried out using a murine infection model showed that some serotypes of *Salmonella* could have invasive characteristics; however, *S.* Panama did not show this ability[17]. However, another study suggested that *S.* Panama is more invasive than *S.* Typhimurium when tested in HEp-2 cells[2]. Indeed, more studies are needed to elucidate the role of the virulence-related genes in the adherence, colonization, and invasiveness of the *S.* Panama serotype.

In the present study, PFGE, which is considered the gold standard typing method for *Salmonella*, was used to assess genetic relatedness (**Figure 1**). The application of the Dice coefficient and an 80% similarity threshold allowed the identification of 18 pulsotypes that were grouped in four clusters: A (24 isolates, 11 pulsotypes), B (1 isolate, 1 pulsotype), C (3 isolates, three pulsotypes), and D (5 isolates, three pulsotypes). Degradation of DNA was an inhibiting factor when typing the isolates by PFGE, and even with the use of high concentrations of thiourea, only 33 XbaI-digested samples could be typed. Cluster A, which consisted of *S.* Panama samples collected from humans, food, the environment, and animal origin (during a period of five years from five different states), showed the dispersal ability and persistence of *S.* Panama. Human isolates (91.0%) were included in this cluster, and the majority showed a similarity of 90%. A more invasive clone was probably the origin of these isolates. In addition, BRJKGX01.011 and BRJKGX01.013 pulsotypes consisted of seemingly unrelated and epidemiologically indistinguishable samples. Cluster B,

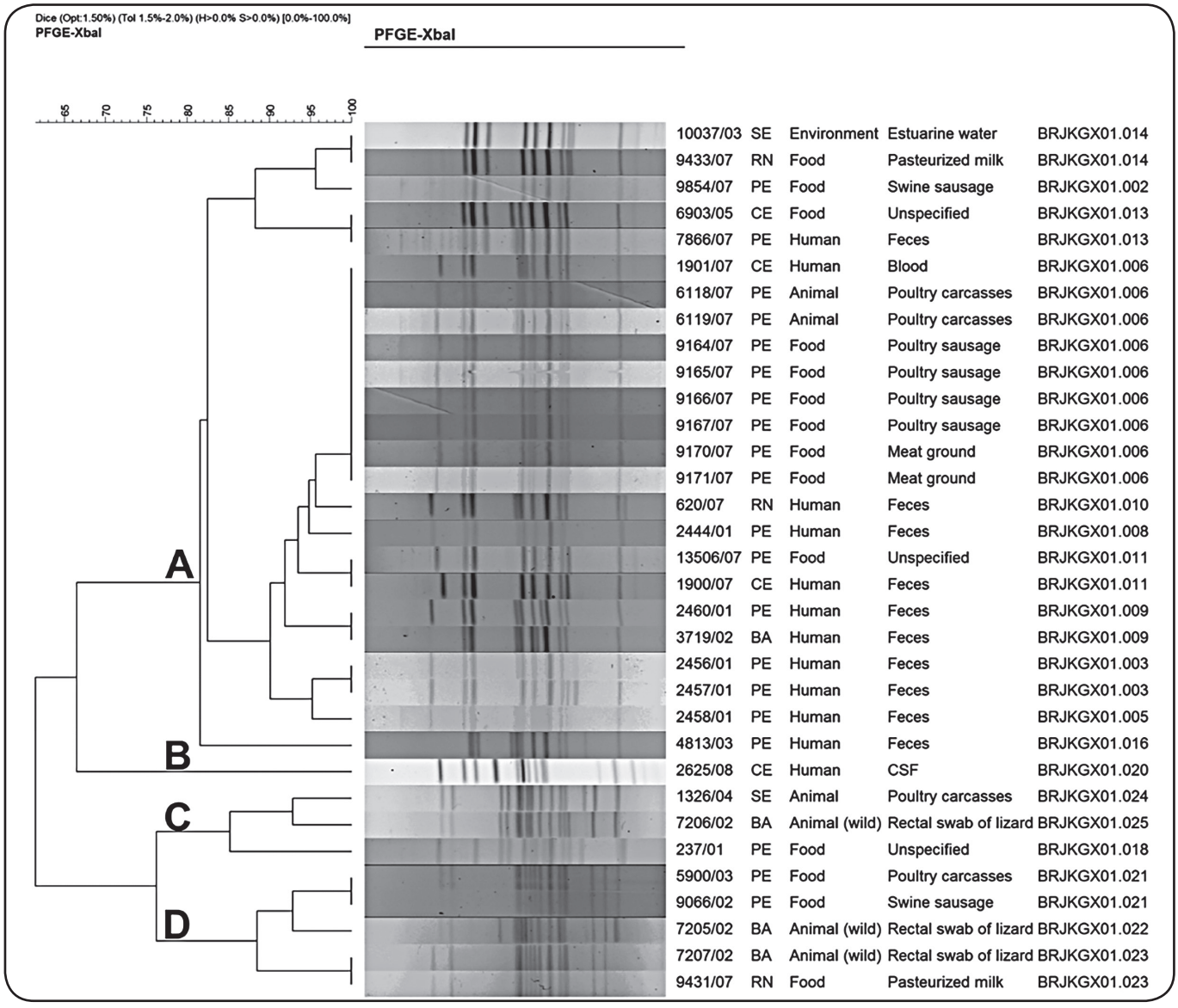

FIGURE1: PFGE profiles of 33 *Xbal*-digested *Salmonella* Panama isolates showing state of origin, source of the isolate, and pulsotypes.

represented by one human isolate, was collected from cerebrospinal fluid (CSF) in 2008. Three isolates belonging to cluster C isolated in Bahia, Pernambuco, and Sergipe, from 2001 to 2004, showed ≥ 85% similarity. The isolates from Bahia (2002) and Sergipe (2004) were from a poultry carcass and a wild animal (lizard), respectively, and showed 92.5% similarity. Two isolates from Bahia, two from Pernambuco, and one from Rio Grande do Norte belonged to cluster D. Two epidemiologically unrelated isolates (one isolated from a wild animal in Bahia in 2002 and the other from pasteurized milk in Rio Grande do Norte in 2007) were placed together and one indistinguishable PFGE pulsotype was observed.

Very little information has been published on the molecular epidemiology of *S*. Panama from Brazil[9]. A study with 110 isolates of *S.* Panama, including isolates from the environment (n=84), humans (n=16), food (n=5), and wild animals (n=5) obtained from different cities in the state of Pará, Brazil reported that 81% of the isolates were typeable by PFGE and a high genetic diversity with 54 distinct profiles and 16 clones was detected. The presence of multiple PFGE profiles was also detected among *S*. Panama isolated from salami[9] in the state of Parana, Brazil. In a study conducted in Taiwan, to investigate the changing serotypes, genotypes and antibiotic resistance of 81 isolates from group D *Salmonella* infection, *S. enterica* serotype Enteritidis was the most common serotype (80%), followed by *S*. Panama (7%); but *S*. Panama was associated with a higher rate of bacteremia and antimicrobial resistance than *S*. Enteritidis. In addition, a PFGE study showed a single genotype of *S*. Enteritidis and diverse genotypes of *S*. Panama[1]. In another study, also conducted in Taiwan, antimicrobial susceptibility testing and molecular typing were performed on nine clinical *S*. Panama isolates using PFGE. Three predominant PFGE types with six subtypes were found among these isolates[16].

This study highlighted the importance of continuous surveillance for detecting the antibiotic resistance profiles and the introduction of new clones into a geographical area, information that can be useful for clinicians in choosing appropriate antibiotics for treating severe group D *Salmonella* infections and should be considered when control programs are planned.

To conclude, our results showed that at the time of this study, the strains of *S*. Panama circulating in the northeast region of Brazil were consistently collected from both humans and non-human sources and shared similar genotypes within each cluster.

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Conflict of interest

The authors declare that there is no conflict of interest.

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