INTRODUCTION

Worldwide, Salmonella is the most common etiological agent of foodborne diarrheal illnesses. There is an increasing concern with this pathogen due to the emergence and spread of antibiotic-resistant and potentially more pathogenic strains. The increase in resistant strains can be attributed to the inappropriate use of antimicrobials as therapeutic or prophylactic agents in human and veterinary medicine, as well as the use of growth promoters in animal production. Studies suggest that the use of antibiotics in food animal production has contributed to the selection of resistant lineages transferable to humans via the food chain. Antimicrobial-resistant Salmonella spp. have been isolated from different foods of animal origin around the world.

The ability of Salmonella to cause disease can be attributed to an array of virulence genes located in the chromosome or in large virulence-associated plasmids. Gene invA in the Salmonella Pathogenicity Island (SPI) codes for the production of proteins from the type III secretion system, related to the invasion of Salmonella into eukaryotic host cells. Many Salmonella serotypes harbor virulence plasmids of varying sizes. The Salmonella plasmid virulence (spv) operon, which consists of five genes (spvRABCD), is important for the intracellular survival and replication of Salmonella, and contributes to the systemic phase of the illness. There are many types of fimbriae that mediate Salmonella intestinal adhesion including type 1 fimbriae (Fim), long polar fimbriae (Lpf), aggregative fimbriae (Agf), plasmid-encoded fimbriae (Pef) and Salmonella Enteritidis fimbriae (Sef). The fim and agf operons are highly conserved among isolates of Salmonella, whereas the sef and pef operons are only found in certain serotypes.

The objective of this study was to evaluate the prevalence of antimicrobial resistance and to detect the virulence-related genes among Salmonella spp. isolated from foods associated or not with foodborne salmonellosis.

MATERIALS AND METHODS

Salmonella spp.: The study included 237 randomly selected Salmonella spp. strains isolated from food sources between 1983 and 2007, belonging to the culture collection of Food Microbiology Laboratory of the Adolfo Lutz Institute, São Paulo, Brazil. The isolates belonged to 51 serotypes and most of them were S. Enteritidis (32%).

PREVALENCE OF DRUG RESISTANCE AND VIRULENCE FEATURES IN Salmonella spp. ISOLATED FROM FOODS ASSOCIATED OR NOT WITH SALMONELLOSIS IN BRAZIL

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SUMMARY

Salmonella is the most common etiological agent of cases and outbreaks of foodborne diarrheal illnesses. The emergence and spread of Salmonella spp., which has become multi-drug resistant and potentially more pathogenic, have increased the concern with this pathogen. In this study, 237 Salmonella spp., associated or not with foodborne salmonellosis in Brazil, belonging mainly to serotype Enteritidis, were tested for antimicrobial susceptibility and the presence of the virulence genes spvC, invA, sefA and pefA. Of the isolates, 46.8% were sensitive to all antimicrobials and 51.9% were resistant to at least one antimicrobial agent. Resistance to more than one antimicrobial agent was observed in 10.5% of the strains. The highest rates of resistance were observed for streptomycin (35.9%) and nalidixic acid (16.9%). No strain was resistant to cefoxitin, cephalothin, cefotaxime, amikacin, ciprofloxacin and imipenem. The invA gene was detected in all strains. Genes spvC and pefA were found in 48.1% and 44.3% of strains, respectively. The gene sefA was detected in 31.6% of the strains and only among S. Enteritidis. Resistance and virulence determinants were detected in Salmonella strains belonging to several serotypes. The high rates of antibiotic-resistance in strains isolated from poultry products demonstrate the potential risk associated with the consumption of these products and the need to ensure good food hygiene practices from farm to table to reduce the spread of pathogens relevant to public health.

KEYWORDS: Salmonella spp.; Antimicrobial resistance; Virulence gene; Foodborne salmonellosis.

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Among the selected strains, 42 were isolated from foods associated with foodborne salmonellosis and 195 from food samples not associated with outbreaks or sporadic cases (Table 1). Among the strains from foods associated with salmonellosis, 14.3% were isolated from beef, 11.9% from poultry, 23.8% from confectionery, 21.4% from eggs and mayonnaise, 9.5% from water, 7.1% from vegetables, 2.4% from dairy products and 9.5% from other products. Regarding the strains obtained from foods not associated with salmonellosis, 27.2% were isolated from poultry, 29.7% from beef, 18% from pork, 11.3% from seafoods, 5.6% from condiments, 2% from milk and derivatives, 1.5% from eggs and mayonnaise, 1.5% from vegetables and 2.6% from other products.

| Serovar            | Associated with foodborne salmonellosis | Not associated with foodborne salmonellosis |
|--------------------|-----------------------------------------|---------------------------------------------|
| Enteritidis        | 35                                      | 40                                          |
| Typhimurium        | 1                                       | 14                                          |
| Agona              | 1                                       | 9                                           |
| Infantis           | 1                                       | 8                                           |
| Brandenburg        | 1                                       | 7                                           |
| Saintpaul          | 1                                       | 2                                           |
| I 6,7,:-           | 1                                       | 3                                           |
| Sandiego           | 1                                       | 3                                           |
| Panama             | --                                      | 11                                          |
| Anatum             | --                                      | 12                                          |
| Bredenez           | --                                      | 7                                           |
| Heidelberg         | --                                      | 6                                           |
| Ohio, Oranienburg and Schwarzenburg | -- | 5 each                                   |
| Madelidia, Mbandaka, I 4, 5, 12: i: - and London, | -- | 4 each                                    |
| Hadar and Derby    | --                                      | 3 each                                      |
| Sentfenberg, Cerro, IV 43:Z4, Z24:,-, Glostrup, Give, I 4, 12: i: - and Rubislaw | -- | 2 each                                    |
| Muenster, I 4, 5, 12: b: -, Javiana, 3, 10: Z: -, Emek, Muenchen, Saphra, Levingstone, I 6, 8: E, H: -, Lexington, Brandenising, 1,4, 5, 12, Berta, Poona, I 3, 10: :1,6, Ababetetuba, I 13, 23: Z: -, Tenessees, I 6,8:Z10:,-, Newport, Paratyphi B and Pomon A | -- | 1 each                                    |
| Total              | 42                                      | 195                                         |

**Table 1**

Number of isolates and serovars of *Salmonella* spp. included in the study

**Determination of the antimicrobial susceptibility profile:** The strains were tested for antibiotic resistance by the plate disc diffusion method, according to the Clinical and Laboratory Standards Institute. The following disks (Oxoid, Basingstoke, UK) were included in the test: ampicillin (10 µg), cefoxitin (30 µg), cephalothin (30 µg), cefotaxime (30 µg), imipenem (10 µg), chloramphenicol (30 µg), amikacin (30 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), trimethoprim-sulphamethoxazole (1.25/23.75 µg) and sulphonamides (300 µg). *Escherichia coli ATCC 25922* was used for quality control. Results were interpreted as recommended by CLSI (2012b).

**Investigation of virulence genes:** The virulence genes *pefA*, *invA*, *sefA* and *spvC* were investigated as described by CORTEZ et al. (2006) with some modifications. DNA was extracted from the cultures using the Wizard® Genomic DNA Purification System Kit (Promega) following the manufacturer’s instructions. The *pefa* gene was investigated by singleplex PCR with the primers *pefa*-1 (5’- TTTCCATTATTGCACCTGTTG-3’) and *pefa*-2 (5’-AAAGCCACTGCGAAAGATGCC-3’)13. The genes *invA*, *sefA* and *spvC* were amplified by multiplex PCR with the following primers: *invA*-1 (5’-TGTACGGCTTAATTTGACAC-3’) and *invA*-2 (5’-CTGACTGCTTACCCCTTGCATG-3’)15; *sefA*-3 (5’-GCAAGCGTTTATTGGACG-3’) and *sefA*-4 (5’-TGTACAGAGACATTTAAGCC-3’)16; *spvC*-1 (5’-GGAATATACATCTATTACAAA-3’) and *spvC*-2 (5’-CCCCAAAACCACTTACTCGT-3’)17.

Singleplex PCR was performed in a reaction volume of 25 µL containing PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl2, pH 8.3), 200 µM dNTP, 3 mM MgCl2, 0.1 µM *pefa* primers, 2.5 U *Taq* DNA polymerase, and 2 µL DNA template. Amplification was carried out in an automatic thermocycler (GeneAmp PCR System 2400, Perkin Elmer) with the following cycles: 94°C for two minutes, followed by 35 cycles of 94°C for 30 seconds, 50°C for 45 seconds and 72°C for one minute, and a final elongation at 72°C for seven minutes. The samples were stored at 4°C until electrophoresis. The same conditions were used for the multiplex PCR, except that primers were used in the following concentrations: 0.2 µM *invA*, 0.5 µM *sefA* and 0.5 µM *spvC*; in addition, annealing temperature was 55°C rather than 50°C. Electrophoresis of amplified products was carried out using 1.5% agarose gel in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing 0.5 µg/mL of ethidium bromide. The amplified DNA fragments were visualized under UV light. *S. Enteritidis ATCC 13076* and *S. Typhimurium ATCC 14028* were used as positive controls.

**RESULTS AND DISCUSSION**

Antimicrobial susceptibility tests indicated that 51.9% of the strains were resistant to at least one antimicrobial agent and that 10.5% were resistant to more than one antimicrobial. Resistant strains were more common in foods not associated with foodborne salmonellosis (55.9%) than in those related to salmonellosis (33.3%). The resistant *Salmonella* spp. were grouped in 14 different antimicrobial resistance profiles (Table 2). Resistance was observed in 35 serotypes, mainly in *S. Infantis* (88.9%), *S. Typhimurium* (80%), *S. Enteritidis* (58.7%), *S. Anatum* (50%) and *S. Agona* (40%).

All *Salmonella* spp. were susceptible to cefoxitin, cephalothin, cefotaxime, amikacin, ciprofloxacin and imipenem. As for other antibiotics, resistance to streptomycin prevailed in strains associated with salmonellosis (16.7%) in comparison with those not associated with salmonellosis (39%). Resistance to streptomycin was observed in strains belonging to 33 different serotypes. Similar rates were reported in Spain, the United States and Brazil. According to PEIRANO et al.
Table 2
Antimicrobial resistance among Salmonella spp. strains isolated from foods associated and not associated with foodborne salmonellosis

| Resistance to          | Associated with foodborne salmonellosis | Not associated with foodborne salmonellosis |
|------------------------|----------------------------------------|---------------------------------------------|
| STR                    | 2 (4.8%)                               | 57 (29.2%)                                 |
| NAL                    | 7 (16.7%)                               | 28 (14.3%)                                 |
| TET                    | --                                     | 3 (1.6%)                                   |
| AMP                    | --                                     | 1 (0.5%)                                   |
| GEN + STR              | 1 (2.4%)                               | 5 (2.6%)                                   |
| GEN + KAN              | --                                     | 1 (0.5%)                                   |
| TET + STR              | --                                     | 6 (3.1%)                                   |
| SSS + STR              | --                                     | 2 (1.0%)                                   |
| GEN + KAN + STR        | 1 (2.4%)                               | --                                          |
| GEN + NAL + STR        | 2 (4.8%)                               | 1 (0.5%)                                   |
| TET + SSS + STR        | --                                     | 2 (1.0%)                                   |
| TET + KAN + STR        | --                                     | 1 (0.5%)                                   |
| TET + NAL + CHL + STR  | --                                     | 2 (1.0%)                                   |
| AMP + STX + SSS + STR  | 1 (2.4%)                               | --                                          |

Number of resistant strains: 14 (33.3%) 109 (55.9%)

AMP, ampicillin; FOX, cefoxitin; CEF, cefalothin; CTX, cefotaxime; CHL, chloramphenicol; TET, tetracycline; AK, amikacin; GEN, gentamicin; STX, trimethoprim-sulphamethoxazole; CIP, ciprofloxacin; NAL, nalidixic acid; IPM, imipenem; KAN, kanamycin; SSS, sulphonamides; STR, streptomycin.

Table 3
Prevalence of the spvC, sefA and pefA genes among Salmonella spp. strains isolated from foods associated and not associated with foodborne salmonellosis

| Serovar          | Strains associated with food poisoning | Strains not associated with food poisoning |
|------------------|---------------------------------------|------------------------------------------|
|                  | n | spvC | sefA | pefA | n | spvC | sefA | pefA |
| S. Enteritidis   | 35 | 33 | 100 | 35 | 100 | 34 | 100 |
| S. Typhimurium   | 1 | 1 | 100 | 0 | 0 | 1 | 100 |
| S. Agona         | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. Infantis      | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. Brandenburg   | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. Saintpaul     | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. enterica 1, 6, 7:r:- | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. Sandiego      | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. Panama        | 0 | n.a. | n.a. | n.a. | 11 | 7 | 63.6% | 0 | 0 |
| S. Anatum        | 0 | n.a. | n.a. | n.a. | 12 | 9 | 75% | 0 | 2 | 16.7% |
| S. Hadar         | 0 | n.a. | n.a. | n.a. | 3 | 2 | 66.7% | 0 | 0 | 100% |
| S. Heidelberg    | 0 | n.a. | n.a. | n.a. | 6 | 1 | 16.7% | 0 | 1 | 16.7% |
| S. enterica 1, 4, 5, 12:i:- | 0 | n.a. | n.a. | n.a. | 4 | 0 | 0 | 0 | 2 | 50% |
| S. Bredeney      | 0 | n.a. | n.a. | n.a. | 7 | 5 | 71.4% | 0 | 0 | 71.4% |
| S. enterica 1, 4, 12:i:- | 0 | n.a. | n.a. | n.a. | 2 | 1 | 50% | 0 | 1 | 50% |

n.a. = non applicable
one of the most frequently used therapeutic drugs in animal production\textsuperscript{41} thus contributing to the high levels of resistance to this antimicrobial\textsuperscript{12}. In this survey, resistance to this antibiotic was low (5.9\%) whereby eight strains were isolated in years prior to the prohibition and six were isolated in 2002 (Table 5) and found in strains belonging to serotypes Typhimurium, Bradenburg, Hadar, I 4, 5, 12: i-, Derby, Bredeney and I 6, 8; Z10: -. The low resistance observed for the sulphonamides is surprising, since other Brazilian studies have reported a high frequency of resistance to this antibiotic in \textit{Salmonella}\textsuperscript{23,30}.

Regarding S. Enteritidis, the serotype with the highest prevalence in humans in Brazil\textsuperscript{19}, 31.4\% (11/35) of the strains associated with foodborne salmonellosis and 82.5\% (33/40) of the strains not involved in salmonellosis were resistant to one or more antimicrobial agents. The higher prevalence of resistance among strains not involved in salmonellosis cases or outbreaks may be related to food sources. Among the strains not associated with foodborne salmonellosis, 81.8\% were isolated from poultry and related products, whereas among the strains involved in salmonellosis, only 21.4\% were obtained from these products. In Brazil, resistant S. Enteritidis has been frequently isolated from foods of animal origin, mainly poultry-related products\textsuperscript{2,16,18,41,32}. Concerning nalidixic acid, a total of 70\% of strains not associated with salmonellosis was resistant to this antibiotic, while 25.7\% of the strains involved in salmonellosis presented this phenotype, certainly due to the higher number of strains not associated with salmonellosis isolated from poultry and poultry-related products. On the other hand, similar resistance to streptomycin and gentamycin was observed in strains associated or not with salmonellosis: 11.4\% and 15\% for streptomycin, respectively, and 11.4\% and 12.5\% for gentamycin, respectively.

The virulence gene \textit{invA} was detected in all strains, which is consistent with the findings from previous reports\textsuperscript{24,40}. \textit{InvA} is conserved among \textit{Salmonella} serotypes and is a useful marker for molecular detection of this pathogen by PCR\textsuperscript{15,31}.

The genes \textit{spvC}, \textit{sefA} and \textit{pefA} were observed in 48.1\%, 31.6\% and 44.3\% of the strains, respectively. Studies have shown a heterogeneous distribution of these virulence factors in \textit{Salmonella} isolated from different origins\textsuperscript{2,13,53,54}.

The \textit{spvC} gene has an important role in systemic infection of \textit{Salmonella}\textsuperscript{24,34} and in the present survey this gene was found in 80.9\% (34/42) and 41\% (80/195) of the strains associated and not associated with foodborne salmonellosis, respectively (Table 3). The \textit{spv} operon is located in virulence plasmids and limited to specific serotypes (i.e. Enteritidis, Typhimurium, Dublin, Choleraesuis, Gallinarum, Pullorum and Abortus-ovis)\textsuperscript{44}. Among the strains associated with salmonellosis, the serotypes that commonly have plasmids (\textit{S. Enteritidis} and \textit{S. Typhimurium}) corresponded to 86\% of the analyzed strains, whereas among those not involved in salmonellosis, these two serotypes corresponded to only 27.7\% of the strains.

The \textit{sefA} gene was detected in 83.3\% (35/42) and 20.5\% (40/195) of the strains associated and not associated with salmonellosis, respectively. This gene was only present in S. Enteritidis (Table 3), which is an expected result since the genes of the \textit{sef} operon (\textit{sefABCD}) are restricted to the serotypes of serogroup O:9, which include S. Enteritidis, S. Typhi, S. Dublin, S. Berta, S. Gallinarum and others\textsuperscript{44}. Due to the specificity of the \textit{sef} gene, it has been used for the molecular identification of \textit{Salmonella} Enteritidis\textsuperscript{10,11,12}.

The \textit{pefA} gene was present in 83.3\% (35/42) and 35.9\% (70/195) of the strains associated and not associated with foodborne salmonellosis, respectively (Table 3). Like the \textit{spvC} gene, \textit{pefA} is located in virulence plasmids, and limited to certain serotypes such as Enteritidis, Choleraesuis and Typhimurium\textsuperscript{1}.

All S. Enteritidis isolated from samples associated or not with foodborne salmonellosis contained the \textit{invA} and \textit{sefA} genes. The prevalence of \textit{invA} and \textit{sefA} genes in strains associated (94.3\% and 97.1\%, respectively) and not associated with salmonellosis (87.5\% and 95\%, respectively) was similar. Regarding these four genes, S. Enteritidis strains evaluated in this study were grouped into three profiles (Table 4), with a predominance of strains in the P1 profile (presence of the four virulence genes). These results highlight the pathogenic potential of S. Enteritidis, which is the major serotype associated with human salmonellosis in Brazil\textsuperscript{19}.

This survey illustrates the occurrence of antibiotic-resistance and a wide distribution of virulence genes in \textit{Salmonella} spp. regardless of their association with foodborne salmonellosis. Resistance and virulence determinants were detected in strains belonging to several serotypes. The high rates of antibiotic-resistance in strains isolated from poultry demonstrate the potential risk associated with the consumption of these products and the need to ensure good food hygiene practices from farm to table in order to reduce the spread of antibiotic-resistant microorganisms from animals to humans.

The development of antimicrobial resistance in zoonotic bacteria, such as non-typhoidal \textit{Salmonella}, constitutes a public health risk with many consequences for human health. As bacteria become increasingly more resistant to drugs of clinical importance, there is an increased risk of treatment failure due to limited therapeutic choices\textsuperscript{59}. Furthermore, antibiotic-resistance and virulence determinants may be present in the

| Profile | Number of isolates | Genes |
|---------|-------------------|-------|
|         | Associated with food poisoning | Not associated with food poisoning | spvC | invA | sefA | pefA |
| P1      | 33 (94.3\%)       | 35 (87.5\%)    | +    | +    | +    | +    |
| P2      | 1 (2.8\%)         | 2 (5\%)        | -    | +    | +    | +    |
| P3      | 1 (2.8\%)         | 3 (7.5\%)      | -    | +    | +    | -    |
Resumo

Prevalência de resistência antimicrobiana e características de virulência em *Salmonella* spp. isoladas de alimentos associados ou não com salmonelose no Brasil

*Salmonella* é o agente etiológico mais comumente envolvido em casos e surtos de doenças diarreicas de origem alimentar. A preocupação com este patógeno é, ainda, maior quando se verifica o surgimento e a disseminação de cepas multirresistentes e potencialmente mais patogênicas. Neste estudo, 237 cepas *Salmonella* spp., associadas ou não com casos ou surtos de salmonelose e pertencentes, principalmente, ao sorotipo *Enteritidis*, foram avaliadas quanto ao perfil de resistência antimicrobiana e presença dos genes de virulência *spvC*, *invA*, *sefA* e *pefA*. Entre as cepas avaliadas, 46,8% foram sensíveis a todos os agentes antimicrobianos e 51,9% foram resistentes a pelo menos uma droga. As maiores taxas de resistência foram observadas para estreptomicina (35,9%) e ácido nalidíxico (16,9%). Não foram detectadas cepas resistentes à cefoxitina, cefalotina, cefoxitamina, ampicilina ou imipenem. O gene *invA* foi detectado em todas as cepas de *Salmonella*. Os genes *spvC* e *pefA* foram encontrados em 48,1% e 44,3% das cepas, respectivamente. O gene *sefA* foi detectado em 31,6% das cepas, estando presente somente entre as cepas de *S. Enteritidis*. Resistência antimicrobiana e marcadores de virulência foram detectados em cepas de *Salmonella* pertencentes a diversos sorotipos. A alta taxa de resistência antimicrobiana verificada em cepas isoladas de frangos e derivados demonstra o potencial risco associado ao consumo destes produtos e a necessidade de se assegurar boas práticas de higiene em toda cadeia produtiva para reduzir a disseminação de patógenos relevantes para a saúde pública.

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467