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QUANTIFICATION AND MOLECULAR CHARACTERIZATION OF *SALMONELLA* ISOLATED FROM FOOD SAMPLES INVOLVED IN SALMONELLOSIS OUTBREAKS IN RIO GRANDE DO SUL, BRAZIL

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ABSTRACT

Data concerning the prevalence and populations of *Salmonella* in foods implicated in outbreaks may be important to the development of quantitative microbial risk assessments of individual food products. In this sense, the objective of the present study was to assess the amount of *Salmonella* sp. in different foods implicated in foodborne outbreaks in Rio Grande do Sul occurred in 2005 and to characterize the isolated strains using phenotypic and genotypic methods. Nineteen food samples involved in ten foodborne outbreaks occurred in 2005, and positive on *Salmonella* isolation at the Central Laboratory of the Health Department of the State of Rio Grande do Sul, were included in this study. Food samples were submitted to estimation of *Salmonella* using the Most Probable Number (MPN) technique. Moreover, one confirmed *Salmonella* colony of each food sample was serotyped, characterized by its *Xba*I-macrorestriction profile, and submitted to antimicrobial resistance testing. Foods containing eggs, mayonnaise or chicken were contaminated with *Salmonella* in eight outbreaks. Higher counts ($>10^7$ MPN.g^{-1}) of *Salmonella* were detected mostly in foods containing mayonnaise. The isolation of *Salmonella* from multiple food items in five outbreaks probably resulted from the cross-contamination, and the high *Salmonella* counts detected in almost all analyzed samples probably resulted from storing in inadequate temperature. All strains were identified as *S*. Enteritidis, and presented a unique macrorestriction profile, demonstrating the predominance of one clonal group in foods involved in the salmonellosis outbreaks. A low frequency of antimicrobial resistant *S*. Enteritidis strains was observed and nalidixic acid was the only resistance marker detected**.**

Key-words: *Salmonella,* foodborne outbreak, quantification, PFGE.

INTRODUCTION

In Southern Brazil, a high prevalence of *Salmonella* isolation has been found in pigs (2), pork (6) and pork products (24). In opposite to that, pork is rarely involved in salmonellosis outbreaks reported in this region (8,25). Data collected in Rio Grande do Sul, during the period of 1997 to 1999, pointed salad prepared with homemade mayonnaise as the most often implicated food in salmonellosis outbreaks, accounting for 42.45% of all identified food vehicles (8). Factors responsible for the discrepancy between *Salmonella* prevalence in pork and the frequency of foodborne outbreaks attributed to pork consumption need to be better investigated.

It is well documented that exposure to larger quantities of foodborne pathogens usually results in a greater risk to human health (14,18,34). Consequently, the final concentration of *Salmonella* in food is an important parameter contributing to overall disease risk. In this sense, data on the prevalence and

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populations of *Salmonella* in foods implicated in outbreaks may be important to the development of quantitative microbial risk assessments of individual products.

Another aspect contributing to the understanding of *Salmonella* epidemiology is the characterization of the strains involved in outbreaks. Serotyping is traditionally conducted as a first approach in the characterization and usually forms the background for all other typing methods (28). Over the past two decades genotyping has been associated to the traditional typing methods to achieve a better discrimination of strains and to identify bacterial clones (28). Among the molecular methods, pulsed-field gel electrophoresis (PFGE) is considered the standard method for DNA fingerprinting in *Salmonella*, and has been performed to investigate salmonellosis outbreaks (11,16,17,21,40).

In this sense, the objective of the present study was to assess the amount of *Salmonella* sp. in different foods implicated in foodborne outbreaks in Rio Grande do Sul occurred in 2005 and to characterize the isolated strains using phenotypic and genotypic methods.

MATERIAL AND METHODS

Food samples

Nineteen food samples involved in ten foodborne outbreaks occurred in Rio Grande do Sul in 2005, and positive for *Salmonella* isolation at Laboratório Central da Secretaria Estadual da Saúde (Central Laboratory of State Health Department, LACEN/RS , Porto Alegre, Rio Grande do Sul) were included in this study. Food samples were stored during the analysis period in sterile flasks at 4ºC. Data available for each confirmed salmonellosis outbreak were obtained from the epidemiological investigation report received with the food samples by LACEN.

Salmonella quantification and serotyping

Food samples were submitted to estimation of *Salmonella* using the Most Probable Number (MPN) technique as previously described (4) with modifications. From each positive sample, 25 g were added to 225 mL of Buffered Peptone Water (BPW). The samples were homogeneized for 1 min (Stomacher, Interscience, St. Nom, France) and decimal dilutions up to 10^{-8} were prepared in BPW. Triplicate tubes of all dilutions were incubated at 35ºC for 18 h. From each dilution tube, aliquots of 0.1 mL were transferred to 9.9 mL of Rappaport-Vassiliadis broth (Merck, Darmstadt, Germany). Following incubation on the selective enrichment media at 42ºC for 24 h, samples were streaked onto XLT4 (Difco, Sparks, USA) agar. After 24 h incubation at 37ºC, suspected colonies from each plate were confirmed as *Salmonella* by biochemical tests and agglutination using poly O-antiserum (Probac, São Paulo, Brazil). The number of tubes in each dilution, from which colonies were confirmed as *Salmonella*, was used to estimate *Salmonella* counts using the MPN table (1). One confirmed *Salmonella* colony of each food sample was serotyped at Fundação Oswaldo Cruz (Brazilian Salmonella Reference Institute, Rio de Janeiro, Brazil).

Macrorestriction analysis

Genomic DNA of one *Salmonella* isolate obtained from each positive food sample was extracted as previously described (22,36). Slices of DNA-containing agarose plugs were digested with 20 units of *Xba*I (Promega, Madison, USA) at 37ºC for 18 h. The respective fragments were separated by pulsed field gel electrophoresis (PFGE) in 1% PFGE-certified agarose gel (BioRad, Hercules, USA) in a CHEF DR II system (BioRad, California, USA) at 5.6 $V.cm^{-1}$ with 0.5 × TBE as the running buffer. In order to avoid the DNA degradation of *Salmonella* isolates, 50 µmol of Thiourea (Acros Organics, Geel, Belgium) was added to the running buffer. The pulse times were increased from 10 to 30 s during the first 11 h and subsequently from 30 to 50 s during the next 13 h. The gel was stained with ethidium bromide (2 mg/mL, Sigma, St. Louis, USA) and photographed under UV-illumination. Patterns produced by PFGE were compared using the GelCompar II software package (Applied Maths, Kortrijk, Belgium).

Antimicrobial susceptibility testing

Antimicrobial resistance was determined by agar disk diffusion tests using disks with the following antimicrobials (Cefar Diagnóstica, São Paulo, Brazil): amikacin (30 µg), ampicillin (10 µg), cefaclor (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), tetracycline (30 µg), tobramycin (10 µg), streptomycin (10 µg), sulfamethoxazole-trimethoprim (23.75/1.25 µg), and sulfonamide (300 µg). The testing was conducted and evaluated according to the document M100-S15 of the Clinical and Laboratory Standards Institute (7). *Escherichia coli* ATCC 25922 was used for quality control testing.

RESULTS

In eight salmonellosis outbreaks analyzed in the present study, a total of 212 people were exposed and 15 patients needed hospitalization. In two outbreaks, epidemiological data regarding the city of origin and/or the number of exposed people were not available (Table 1). The major symptoms observed were fever, diarrhea and abdominal pain, while nausea and vomiting were reported in six outbreaks. The median incubation period varied from 9 to 24 hours, and in most part of the outbreaks, symptoms appeared between 13 and 17 hours after the food ingestion (data not shown).

Foods containing eggs, mayonnaise or chicken were contaminated with *Salmonella* in eight outbreaks (Table 1).

Table 1. *Salmonella* quantification in foods involved in outbreaks in Rio Grande do Sul, Brazil, in 2005.

Outbreak#	City	Date (month/day)	Number of exposed	Number of hospitalizations	Involved food	*Salmonella* serovar	*Salmonella* quantification MPN.g^{-1}
1	Alecrim	01/24	5	0	Potatoes with mayonnaise	Enteritidis	$4.6x10^9$
2	Camaquã	02/21	5	0	Chicken	Enteritidis	$1.1 x10^5$
					Cooked beet	Enteritidis	$2.4x10^6$
3	Frederico Westphalen	04/19	10	0	Cake	Enteritidis	$2.4x10^5$
4	Teutônia	04/23	9	1	Potatoes with mayonnaise	Enteritidis	$2.4x10^7$
					Sausage	Enteritidis	$3.6x10^7$
					Chicken	Enteritidis	$4.6x10^6$
					Pork	-	<3
5	Ipê	04/25	6	3	Cassava with mayonnaise	Enteritidis	$2.8x10^8$
					Roasted beef	Enteritidis	$4.3x10^3$
					Turkey	-	<3
6	ND	05/15	ND	ND	Chicken	-	<3
					Roasted beef	Enteritidis	$4.6x10^5$
7	São Pedro da Serra	10/16	160	3	Potatoes with mayonnaise	Enteritidis	$1.1x10^8$
8	Santa Rosa	11/27	ND	ND	Cake	Enteritidis	$1.5x10^8$
9	Palmeira das Missões	11/27	15	8	Cooked pea	Enteritidis	$1.1x10^3$
					Minced meat	Enteritidis	$3x10^2$
					Tomato	-	<3
10	Porto Alegre	12/28	2	0	Salami	-	<3

Higher counts (>10^7 MPN.g^{-1}) of *Salmonella* were detected mostly in foods containing mayonnaise. Other food items with high counts were the cake in outbreak #8, and the pork sausage in outbreak #4. In five outbreaks more than one food item were positive on *Salmonella* isolation. In these cases, food items with low as well as high *Salmonella* counts were detected in a same outbreak.

All fourteen *Salmonella* strains submitted to serotyping were classified as *S.* Enteritidis and resulted in a single PFGE profile (Fig. 1). Six *S.* Enteritidis strains were resistant only to nalidixic acid, while the remaining eight strains were sensible to all tested antimicrobials (data not shown).

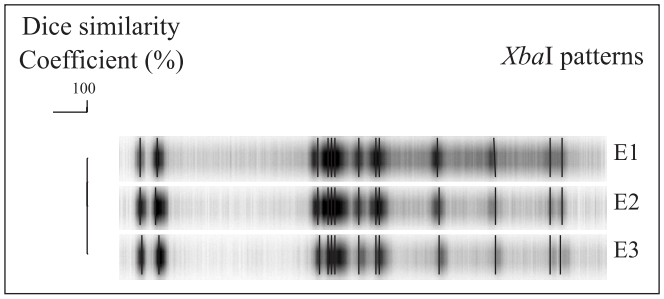

Figure 1. *Xba*I-macrorestriction profiles identified in *Salmonella* Enteritidis strains isolated from foods involved in outbreaks in Rio Grande do Sul, Brazil in 2005.

DISCUSSION

In the present study we analyzed foods collected in ten confirmed salmonellosis outbreaks investigated in Rio Grande do Sul in the year 2005. These outbreaks probably represent a small fraction of all salmonellosis cases that occurred in this region, since the lack of reporting to the sanitary authority and failure on identification of the responsible food is not a rare event in foodborne disease surveillance (35).

Salmonellosis has been the most important foodborne disease, in Rio Grande do Sul, since 1993 (8,25). In most salmonellosis outbreaks investigated in Brazil (8,25,30) and in

other countries (3,29), foods prepared with eggs were implicated as the *Salmonella* vehicle. Similarly, in the present study mayonnaise and cakes are among the foods with *Salmonella* isolation in six outbreaks, and together with chicken are implicated in all except two outbreaks analyzed. In contrast to that, only in outbreak #4 *Salmonella* was isolated from pork and pork sausage. However, *Salmonella* has been frequently isolated from pigs and pork (2,6,19,24), demonstrating that this pathogen circulates throughout the swine production chain. Possible reasons for these conflicting results are the low *Salmonella* counts found in pork products sampled in Rio Grande do Sul (24), and the habit of preparing pork products well-done, allowing the destruction of any *Salmonella* that eventually is present in the food.

The isolation of *Salmonella* from multiple food items in five outbreaks suggests the cross-contamination due to improper manipulation. Moreover, the high *Salmonella* counts detected in almost all analyzed samples probably resulted from storing foods at ambient temperature for more than two hours or by their inadequate refrigeration. This kind of processing failures has been commonly pointed among the factors contributing for salmonellosis outbreaks in Rio Grande do Sul (8,25). The association of cross-contamination and storing in inadequate temperature can explain the *Salmonella* counts found on the cooked beet and on the cooked peas in outbreaks #2 and #9, respectively. In both outbreaks, animal derived products were also involved, thus the contact with raw animal-derived foods as well as with contaminated surfaces in the kitchen may have allowed the cross-contamination of the cooked foods. Afterwards, the holding of the contaminated cooked foods at ambient temperature permitted the multiplication of *Salmonella,* which attained high population counts prior the consumption.

The number of involved in an outbreak and the severity of the symptoms are related to susceptibility of the individuals (38), food composition and *Salmonella* number on the food (13). Moreover, persons who had eaten higher amounts of contaminated foods were found more likely to have shorter incubation periods and higher hospitalization risk (14). Considering that, an infecting dose between 10^5 and 10^7 colony forming units (cfu) is usually accepted for immunocompetent adults (38). Most foods analyzed in the present study had *Salmonella* counts above the proposed infecting dose.

All *Salmonella* strains analyzed were serotyped as Enteritidis, demonstrating the predominance of this serovar in foods involved in salmonellosis outbreaks occurring in Rio Grande do Sul in the year 2005. Previous studies reported that *S*. Enteritidis has become the main cause of salmonellosis outbreaks (9,10,12,23,30,31) and this serovar has been also the most prevalent among poultry-related samples in Brazil (32,37). Furthermore, a high adaptation of *S*. Enteritidis to colonize the chicken oviduct has been proposed, indicating that poultry may be an important reservoir of this pathogen (20).

In spite of being isolated from not related outbreaks, all strains characterized in this study presented a common PFGE pulse-type, demonstrating a clonal relationship among them. In previous studies conducted in Rio Grande do Sul (26,39), strains isolated from poultry and humans have also demonstrated a clonal relationship, which might contribute to elucidate the origin of salmonellosis cases. Since strains from poultry were reported as clonal due to the possible spread of one clone by international trade of breeding lines (33), a common clone causing foodborne outbreaks transmitted by egg products is not unexpectedly. Moreover, predominant PFGE patterns are also found in strains isolated from outbreaks in other countries (17) indicating that common clones may be responsible for the disease cases. Such results reinforce the limited diversity of *S*. Enteritidis strains, and point to the need of further studies to investigate other characteristics of these clones such as survival and colonization capability.

A low frequency of antimicrobial resistant *S*. Enteritidis strains was observed and nalidixic acid was the only resistance marker detected. A shift in the resistance level was observed in *S*. Enteritidis strains isolated in the same region over time (5,27,39). In general, antimicrobial resistance was higher in samples of animal origin than those related to food products, demonstrating the high selective pressure caused by the use of antimicrobials in the food production chain. However, also in poultry samples a reduction on the frequency of resistant strains could be found after 1998, when many antimicrobials were banished for growth promotion in Brazil (39). The reduction of antimicrobial use, occurred in the poultry production chain in recent years, may explain the low frequency of resistance that we found in our study.

On the other hand, the resistance to nalidixic acid reflects the still widespread use of this antimicrobial in poultry therapy in Brazil and other countries. It is a matter of concern, since emergence of resistance to nalidixic acid has been associated to decreased susceptibility to fluoroquinolones, which are used for treatment of human patients suffering of severe salmonellosis cases (15). The prevalence of these strains in foods involved in outbreaks represents a risk of salmonellosis cases caused by strains resistant to the drug used in therapy.

In conclusion, high counts ($>10^3$ MPN.g^{-1}) of *Salmonella* were detected in most foods implicated in the reported salmonellosis outbreaks occurred in 2005 in Rio Grande do Sul. Our results suggest that one PFGE clonal group of *S*. Enteritidis was involved in all those salmonellosis outbreaks.

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RESUMO

Quantificação e perfil molecular de *Salmonella* isolada de alimentos envolvidos em surtos de salmonelose no Rio Grande do Sul

Dados sobre a prevalência e a população de *Salmonella* em alimentos implicados em surtos podem contribuir na condução de análises de risco. Dessa forma, o objetivo desse estudo foi determinar a quantidade de *Salmonella* sp. presente em alimentos implicados em surtos ocorridos no Rio Grande do Sul em 2005 e caracterizar os isolados por meio de técnicas fenotípicas e genotípicas. Dezenove amostras de alimentos obtidas em dez surtos ocorridos em 2005 e identificadas como positivas para *Salmonella* no Laboratório Central da Secretaria da Saúde do Estado do Rio Grande do Sul foram incluídas no estudo. A quantificação de *Salmonella* foi feita pela técnica do Número Mais Provável (NMP). Ao lado disto, uma colônia de *Salmonella* obtida de cada amostra de alimento foi submetida à sorotipificação, macro-restição com *Xba*I e determinação de resistência a antimicrobianos. *Salmonella* esteve presente em alimentos a base de ovos, maionese e frango em oito surtos. As contagens mais elevadas ($>10^7$ NMP.g^{-1}) foram detectadas principalmente em alimentos contendo maionese. O isolamento de *Salmonella* de vários alimentos em cinco surtos resultou, provavelmente, da contaminação cruzada, enquanto as elevadas contagens encontradas, em quase todos os alimentos, podem ser explicadas por armazenamento em temperatura inadequada. Todos os isolados foram identificados como *S.* Enteritidis, e apenas um perfil foi encontrado na macro-restrição, demonstrando a predominância de um grupo clonal desse sorovar nos surtos de salmonelose. Uma baixa freqüência de isolados de *S.* Enteritidis resistentes a antimicrobianos foi encontrada, sendo a resistência ao ácido nalidíxico o único perfil encontrado.

Palavras-chave: *Salmonella*, surtos, quantificação, PFGE

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