

RESEARCH

COAGULASE POSITIVE ENTEROTOXIGENIC *STAPHYLOCOCCUS AUREUS* COUNT IN PARAGUAY SEMI-HARD CHEESE

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Bigeon Giselda*, Brusa Victoria, Pellicer Karina, Copes Julio

Facultad de Cs. Veterinarias Universidad Nacional de La Plata, 60 y 118 CC 296 CP1900 La Plata, Buenos Aires. Argentina.

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CORRESPONDENCE AUTHOR: Bigeon Giselda

E-MAIL: bigeong@hotmail.com;

CONFLICTS OF INTEREST

THERE ARE NO CONFLICTS OF INTEREST FOR ANY OF THE AUTHORS.

ABSTRACT:

Background: One of the foodborne diseases of high incidence in the Latin American region is Staphylococcal intoxication, due to food contaminated with enterotoxins produced mainly by *Staphylococcus aureus*. The aim of this study was to enumerate, isolate and characterize *S. aureus* enterotoxigenic in Paraguayan cheeses obtained at markets in Paraguayan cities.

Methods: Three hundred eighty-two cheese samples were analyzed according to ISO 6888-1. Biochemical test, enterotoxin production ability and genotype characterization by PCR multiplex were performed to isolate.

Results: Eight point thirty-seven percent of samples contained typical colonies of coagulase-positive *S. aureus*. Fourteen strains had the gene encoding enterotoxin A.

Conclusion: The presence of coagulase-positive *S. aureus* in the cheese samples revealed a latent state of staphylococcal food poisoning outbreaks in Paraguay. Type A enterotoxigenic *S. aureus* is the most frequent isolation in this type of products, and multiplex PCR method is an effective and fast identification technique.

KEYWORDS: enterotoxins, foodborne diseases, PCR, Staphylococcal intoxication

INTRODUCTION

Foodborne disease are one of the most concerning public health problem all over the world (World Health Organization, WHO). Hundreds of foodborne disease cases are received by WHO, and the most common causes are food that suffered biological contamination, mainly in developing countries [1]. Twenty percent of food poisoning worldwide is produced by enterotoxigenic *Staphylococcus aureus*. Latin America and Europe reported one of the highest incidence rates [2].

The main *S. aureus* virulence factors are Staphylococcal enterotoxins (SEs). SEs are low weight (26000 - 34000 Daltons) and thermo-tolerant proteins. Twenty-one SEs and enterotoxin-like (SEI) types have been described [3]. SEA (Staphylococcal enterotoxins type A) is the most frequent type associated with food poisoning and in decreasing incidence order were reported SEC1, SEB, SED y SEE [4].

Staphylococcal food poisoning is produced by the consumption of foods containing sufficient amounts of one (or more) preformed enterotoxin [5]. The minimum infective dose is 1 mg of SE, and is produced when *S. aureus* count is up 100.000 colony unit forming (CUF) g⁻¹ of food. The ingestion of 100-200 ng of SE could cause symptoms in susceptible patients [6]. Therefore, low charge of enterotoxigenic *S. aureus* could produce disease [7].

S. aureus is found in nasopharynx and skin of 20-30 % of healthy population [8]. Clinically healthy individuals may contaminate raw materials [9], finished product, equipments and furniture, throw sneezing, coughing and expectoration, or due to skin contact with this surfaces [10]. The presence of *S. aureus* on processed food could be introduced by manipulators, mainly due to failure of good manufacture practices or contaminated raw materials [11].

The presence of *S. aureus* in food is owing to high resistance to low humidity and presence as normal flora in the skin of people and pets [6]. The foodstuffs most frequently associated with *S. aureus* outbreak are milk and dairy products [12], products made with cattle, avian and fish meat, sliced cooked meat, mayonnaise, pastries products, sandwiches and salads [3]. *S. aureus* proliferation in food is improved by different factors, such as storage temperature >10 °C, activity water $> 0, 86$ and $\text{pH} > 5$ [13]. Climate conditions of Paraguay and the intrinsic properties of these foodstuffs, often improve environmental conditions for the development and multiplication of *S. aureus*. Paraguay semi-hard cheese is one of the dairy foodstuffs most frequently consumed in that country. The Paraguay cheese found on the market mainly proceeds from farm-producers.

The aim of the present work was to count, isolate and characterize *S. aureus* from Paraguay semi-hard cheese obtained in retail stores in Paraguay, with the purpose of assessing the risk they represent to public health.

MATERIALS AND METHODS

Three hundred eighty-two samples of Paraguay semi hard cheese were randomly collected from markets of six Paraguayan cities during 2010. One hundred and ninety five samples from San Lorenzo city, sixty five from Asuncion, forty four from Capiata, thirty six from Curuguaty, twenty one from Pilar and twenty one from Villa del Rosario were obtained. Each sample was taken in sterile form a sample of 50 g and were carried refrigerated at 4°C to the laboratory for further analysis.

There are no official standard criteria for this type of food in Paraguay. The samples were analyzed using microbiological criteria for *S. aureus* stipulated by the Código Alimentario Argentino (CAA) [14] (table 1). ISO 6888-1 Standard Method [15] was used for counting and isolation of *S. aureus*. Samples were analyzed within 24 hours (h) from the sampling, in the Laboratory of Microbiology Food SENACSA, Paraguay. From appropriate dilutions (10^{-2} , 10^{-3} and 10^{-4}), 0.1ml were inoculated in duplicate by pour plate procedure in Baird Parker agar (Biokar Diagnostics, Zac de La, France) [16]. Plates shown between 30 and 300 colonies were counted. The results were expressed in gr^{-1} of food. Then the characterization of coagulase positive *S. aureus* was performed.

Phenotypic and genotypic characterization of *S. aureus* strains were conducted at the Laboratory of Food Microbiology, Faculty of Veterinary Sciences of the National University of La Plata, Buenos Aires, Argentina. Baird Parker agar was used to count. After incubation, five typical

and five atypical colonies from each plate were selected to proceed with isolation. Potassium tellurite reduction and lecithinase production of each strain on plate was evaluated for phenotypic characterization. There were performed the following biochemical tests: catalase, coagulase on tube, deoxyribonuclease plate (Neogen Corporation, Lansing, Michigan), production of acetoin in Red broth Methyl / Voges Proskauer (Britania SA, Los Patos, Argentina) and nitrate reduction in brain heart broth (Biokar) with 2.5%, 0.1% KNO_3 (Standard, USA). For sugars fermentation was used phenol red broth and sugars 1% (Becton Dickinson and Co., Sparks, MD, USA). The sugars tested were: mannitol (Standard), maltose (Standard), trehalose (Becton Dickinson and Co.), lactose (Becton Dickinson and Co.) and mannose (Inc. ICN Biomedicals, Ohio, USA). In order to determine the production of enterotoxins A, B, C1, C2, C3, D and E of *S. aureus*, TECRA immunoassay test (International PtyLtd, Australia) was performed.

Table 1. Acceptance microbiological criteria for medium humidity cheese of CAA (36% <humidity <46%)

Microorganisms	Acceptance microbiological	ICMSF ⁵ category	Assay method
Coagulase-positive <i>S. aureus</i> /g ²⁰	$N^1 = 5$ $c^2 = 2$ $m^3 = 100$ $M^4 = 1000$	5	FIL 145:1990 ⁶

1: number of samples analyzed; 2: maximum number of sample units whose results may be between m and M; 3: maximum level of the microorganism in the food, for acceptable quality; 4: maximum level of microorganisms in food for a provisionally acceptable quality; 5: International Commission on Microbiological Specifications for Foods; 6: International Dairy Federation.

Two polymerase chain reactions (PCR) were used to detect *sea*, *seb*, *sec*, *sed*, and *see* and 16S rRNA genes [17]. DNA extraction of isolates was performed by boiling one ml of culture broth incubated 18 h at 37 ° C in triton. The PCR protocol for detecting *sea*, *seb*, *sec* and 16S rRNA genes was performed in a final volume of 50 uL containing: 0.4 mM dinucleótidostrifosfato mix (dATP, dCTP, dGTP and dTTP) (Thermo Scientific, USA), 4 mM MgCl_2 + (Thermo Scientific), 0.3 pM of each primer forward (F) and reverse (R) *sea*, *seb* and *sec*, 0.6 pM 16S rRNA primers F and 16S rRNA R, 0.06 pM Taq DNA polymerase (Thermo Scientific), 1X buffer 5UL (Thermo Scientific) and 5 uL of DNA extract. The same concentrations were used for PCR performed with primers *sed*, *see* and 16S rRNA. The amplification program for both PCR consisted on: denaturation at 95 ° C for 5 minutes (m), 14 cycles

of denaturation at 95 ° C for 30 seconds (s), annealing at 68 ° C for 30 s and extension at 72 ° C for 30 s, 19 cycles of denaturation at 95 ° C for 30 s, annealing at 60 ° C for 30 s and extension at 72 ° C for 30 s, and a final extension at 72 ° C for 2 m. Amplification products were separated by electrophoresis in 2.5% agarose gel EDTA 1X tris acetate buffer, at 100 volt for 30 m.

RESULTS AND DISCUSSION

Three hundred eighty-two samples of cheese were analyzed according to the criteria established by the CAA for *S. aureus*, and thirty two (8.37%) did not comply with the criteria. The results shown that 1.53% (1/65), 4.54% (2/44), 4.76% (1/21), 10.76 % (21/195) and 19.49% (7/36) of the samples from Asunción, Capiata, Pilar, San Lorenzo and Curuguaty city, respectively, not accomplished *S. aureus* criteria for cheeses from CAA (Table 2). It was observed that all samples of Villa del Rosario met this requirement.

Table 2. *Staphylococcus* coagulase positive strains from samples tested

City	Samples	Samples with isolation	% positive
Asunción	65	1	1.53
Capiata	44	2	4.54
Curuguaty	36	7	19.44
Pilar	21	1	4.76
Villa del Rosario	21	0,0	0.0
San Lorenzo	195	21	10.76

From thirty two (8.37%) samples of cheese that did not satisfy the criteria established by the CAA for *S. aureus*, all presented colonies characteristics of *Staphylococcus* spp. From each sample, ten typical and ten atypical colonies were isolated from Baird Parker agar. Seventy two *S. aureus* strains were obtained from these samples.

All samples that did not satisfy the CAA had at least one (40.62%) *S. aureus* strain, characterized as typical by biochemical tests, positive for *16S* gene, and negative for *seb*, *sec*, *sed* and *see* genes. From twelve samples (12/32), fourteen strains that showed the gene encoding enterotoxin A, and also positive for toxin production by immunoassay TECRA test were isolated. The data obtained is consistent with the reported in the literature, which states that the most frequently informed

enterotoxigenic strains are producing enterotoxin A [18,19].

The implemented multiplex PCR offered a new alternative coinciding as described in the literature [20] in terms of efficiency and speed to provide the result of the identification of enterotoxigenic *S. aureus*. This method proved to be a specific and sensitive technique for detection of *Staphylococcus* spp. and genes encoding their enterotoxins.

CONCLUSIONS

It can be concluded that enterotoxigenic *S. aureus* type A is not the only indicator for safety evaluation of this kind of food. *S. aureus* type A is not the only one that can cause food poisoning but it is a microorganism frequently isolated in this type of product and has a great public health impact. Therefore, the isolation of coagulase positive *S. aureus* from samples tested, could indicate a potential source of staphylococcal poisoning outbreaks in Paraguay, due to the consumption of this cheese is widespread in that country.

AUTHOR'S CONTRIBUTIONS

Pellicer Karina and Copes Julio performed sampling procedure and processed samples in the Laboratory of Microbiology Food SENACSA, Paraguay. Bignon Giselda and Brusa Victoria performed phenotypic and genotypic characterization of strains at the Laboratory of Food Microbiology, Faculty of Veterinary Sciences of the National University of La Plata, Buenos Aires, Argentina. All authors contributed in drafting the manuscript.

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