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DETECTING SOURCES OF *STAPHYLOCOCCUS AUREUS* IN ONE SMALL-SCALE CHEESE PLANT IN NORTHWESTERN MEXICO

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ABSTRACT

The study aimed to identify the sources involved in the transference of *Staphylococcus aureus* along the chain production of pasteurized Queso-Fresco (QF) in one cheese-processing plant (CPP) in Northwestern Mexico. Eighty-six samples were collected in the CPP from QF, worker's hands, raw milk, dairy-products and utensils. The Bacteriological Analytical Manual was used to detect and enumerate *S. aureus*, and the Pulsed-Field-Gel Electrophoresis to genetically relate the strains. *S. aureus* was found in 18.6% (16/86) of the samples; raw milk (20%), utensils (13.6%) and hands (10.5%) were identified as bacterial sources reflecting its high prevalence (55.6%) and levels in QF samples, which differs from the Mexican legislation (<3.0 Logcfu/g). The genotypic analyses showed nine *SmaI*-patterns; one of them represented a predominant clone in QF samples, and the remaining profiles suggested the existence of multiple contamination sources at the CPP. Food handlers were the main carriers of bacterium causing final QF contamination.

PRACTICAL APPLICATIONS

The number of small-scale cheese-processing plants in Northwestern Mexico has increased during the past decade. Combining traditional cheese making and good hygiene practices is a challenge, since several human pathogens pose hazard in the product. *S. aureus* is an important food poisoning pathogen that can be transmitted via raw milk and/or via hands to cheese. Mexican population regularly consumes handcrafted cheeses, and there is a lack of information on health issues. Surveillance safety programs complemented by the use of molecular tools allow tracing bacterial contamination, and might aid to correct the cheese-processing plants, also improve the design of adequate hygienic and manufacturing practices to accredit the Queso-Fresco safety and health regulation.

INTRODUCTION

Staphylococcus aureus is a commensal and opportunistic pathogen that cause wide spectrum of infections (Kadariya *et al.* 2014.), being the staphylococcal food poisoning the commonest. Staphylococcal food poisoning results from the consumption of foods containing the staphylococcal enterotoxin preformed by the bacteria (Le Loir *et al.* 2003; Hennekinne *et al.* 2012). Symptoms have a rapid onset (2–4 h), including nausea, vomiting and abdominal cramps, with or without diarrhea. Although the disease is self-limiting, it turns severe in children, elderly or vulnerable population (Hennekinne *et al.* 2012). In the United States, *S. aureus* is a significant cause of foodborne disease causing >241,000 staphylococcal food poisoning cases per year (Scallan *et al.* 2011). Most of staphylococcal food poisoning cases are mainly associated with the consumption of cheeses contaminated with the pathogen (Kousta *et al.* 2010). The incidence of staphylococcal food poisoning could be underestimated as a consequence of misdiagnose and lack of routine surveillance of *S. aureus* and/or its enterotoxins (Kadariya *et al.* 2014).

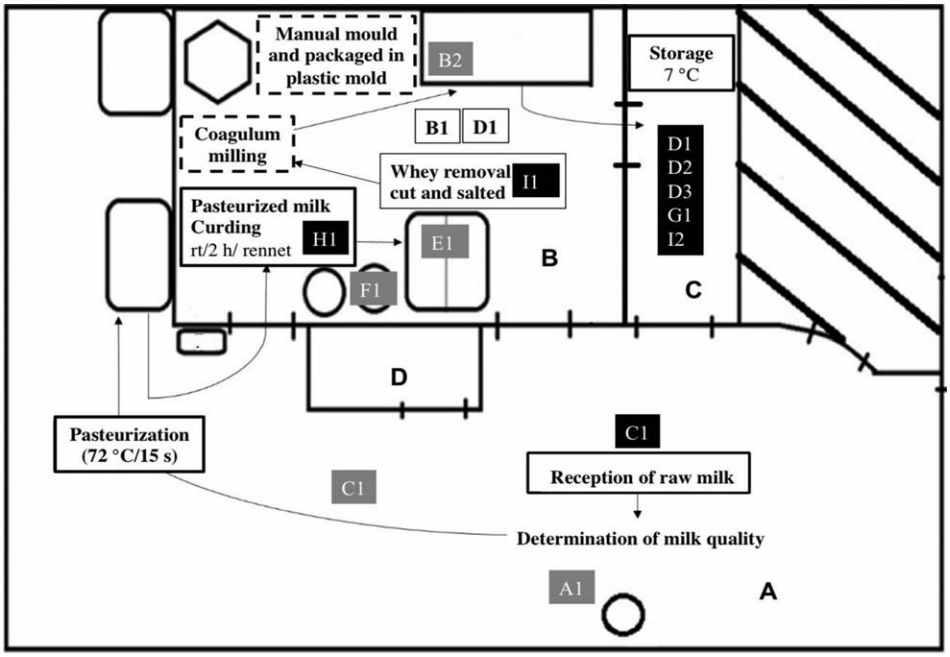

FIG. 1. CHEESE PROCESSING PLANT (CPP) DIAGRAM AND *Staphylococcus aureus* CLONES DISTRIBUTION
Arrows indicate the flow of raw to pasteurized milk, to cheese vat to form the curd, then to milled to manual mold, and then to the cold storage room. The thick outline text chart point out the critical control points of the process, and broken outline text charts indicate direct manual work. Gray, black and white squares meaning the *S. aureus* type recovered from food contact surfaces, food and hands of workers respectively. The nomenclatures of A–I represent the different Pulsed Field Gel Electrophoresis (PFGE) pattern of *S. aureus* recovered at CPP (Table 1).

It's been demonstrated that *S. aureus* may be present in processing plant environment and in cheese final product by mastitis-infected cows or by improper food handler's manipulation (André *et al.* 2008). The lack of hazard analysis of critical control points (HACCP) and Good Manufacturing and Hygiene Practices (GMHP) in the cheese production process, consequently increase the risk of survival and outgrowth of the pathogen (Kousta *et al.* 2010). The robustness of adaptive characteristics and growth of *S. aureus* in food increases the risk of staphylococcal enterotoxins production; because the bacteria can be eliminated by thermal process but the toxins may remain and cause diseases (Schellin *et al.* 2011). The diagnostic criteria for staphylococcal food poisoning are based upon the detection and recovery of 10^5 cfu/g of *S. aureus* (Hennekinne *et al.* 2012, Kadariya *et al.* 2014). These facts make it imperative the mandatory search of *S. aureus* in food, as well as the implementation and monitoring of HACCP and GHP.

Queso-Fresco (QF) is considered a basic ingredient of the Mexican food cuisine; therefore it is the most popular type of cheese consumed with a nationwide production over 45,000 tons/annually (SIAP 2013). The popularity of QF does not coincide with the scarce or null reports of disease related to cheese consumption, including *S. aureus* infections. One possible explanation, but not conclusive, is that *S. aureus* is not mandatory listed in the Mexican National Epidemiological Center (DGE 2015). However, during 2015, the DGE reports >3,800,000 gastrointestinal infections and 25,095 bacterial food poisoning cases with unknown etiological agent (http://www.epidemiologia.salud.gob.mx). Also, in 2015, nearly 148 people were food poisoned by the consumption of QF in Sinaloa State of Mexico, but the etiological agent was unconfirmed (Arias 2015).

To observe the safety of QF, two Standards Health Regulations are in place by the Mexican Authorities; (1) The NOM-251-SSA1-2009 that establishes the requirements for good hygiene practices needed to be observed through the food chain; and (2) The NOM-243-SSA1-2010, that states the health and sanitary specifications to ensure cheese safety. The regulation requires the use of pasteurized milk and declares the permissible limits of *S. aureus* ($\leq 10^3$ cfu/g), total coliforms ($\leq$100 cfu/g) and *E. coli,* ($\leq$100 cfu/g), and the absent of *Salmonella*, *L. monocytogenes*, *Vibrio cholerae*, staphylococcal and botulinum toxins in 25 g of QF tested. However, most QF is still produced with unpasteurized milk, especially among small factories. Fuentes-Coto *et al.* (2013) demonstrated high levels of *S. aureus* in raw milk using to produce QF, posing risk to the consumers. Added to this, Toribio *et al.* (2014) found high levels ($\leq 10^3 - 10^6$ cfu/g) of *S. aureus* on cheese sold in public markets.

TABLE 1. DETECTION AND QUANTIFICATION OF *STAPHYLOCOCCUS AUREUS* AT THE CHEESE PROCESSING PLANT DURING VISITS I AND II

Type	Sample	Samples (*n*)	Positive *S. aureus* samples/*n* (%)		
			After sanitation (I)	QF production (II)	logcfu[abc]
Food contact surface	Raw milk vat	4	0/1	0/3	0
	Raw milk bomb	4	0/1	1/3 (33.3)	2.6
	Hose	4	0/1	1/3 (33.3)	4.3*
	Pasteurized vat	4	0/1	1/3 (33.3)	3.4*
	Knives	4	0/1	1/3 (33.3)	2.2
	Agitators	4	0/1	1/3 (33.3)	2.0
	Mills	4	0/1	0/3	0
	Molding tables	4	0/1	1/3 (33.3)	2.0
	Molds	4	0/1	0/3	0
	Cool room	4	0/1	0/3	0
	Sewer	4	0/1	0/3	0
Workers	Hands	19	0/6	2/13 (15.4)	2.3–3.7*
Raw milk	Supplier 1	3	NT	1/3	0
	Supplier 2	2	NT	1/2 (50.0)	3.9
Dairy products[a]	Pasteurized milk	3	NT	0	0
	Whey	3	NT	1/3 (33.3)	5.8*
	Curd	3	NT	1/3 (33.3)	3.3*
QF[b]	QF	9	NT	5/9 (55.5)	6.5–6.7*
	Total	86	0/17	16/69 (23.2)	

Counting range of *S. aureus* among samples types: [a]logcfu/mL. [b]logcfu/g. [c]logcfu/cm^2.
*Samples over the permissible official limits establishes by NOM-243-SSA1-2010 (>3.0 logcfu/g of QF).
NT, not tested; QF, Queso-Fresco.

Detection of *S. aureus* in cheese reflects poor hygiene practices along the food chain and the risk to acquire staphylococcal food poisoning is high, resulting in the need to identify bacterial contamination sources, and design prevention measures and intervention strategies at processing cheese plant level (Tondo *et al.* 2002). Pulsed Field Gel Electrophoresis (PFGE), is a genetic discriminatory tool that enables to segregate bacterial lineages and can be used to track and correlate those lineages with a specific or multiples origins (MacDougal *et al.* 2003). André *et al.* (2008) identified that raw milk is the main source of *S. aureus* contamination of cheese in a cheese-processing plant (CPP). PFGE was able to depict similarities between strains isolated from QF, and those from raw milk. Also, by means of PFGE analyses, the hands of workers and contact surfaces were also identified as vehicles in the transfer of *S. aureus* to foods by means PFGE analyses (Tondo *et al.* 2002; Lee *et al.* 2012).

Since QF is handcrafted, the likelihood of microbial contamination is high; thus the traceability of microbial contamination source in a CPP is a strategy that allows identifying places most likely harboring pathogenic microorganisms, as well as defining the effectiveness of the sanitation process and personal hygiene. Therefore, the main objectives of this study were to determine the presence of *S. aureus* in the pasteurized cheese-making process, and by PFGE, to investigate the genetic relatedness among the strains.

MATERIALS AND METHODS

Description of the CPP and Cheese Process

The selected CPP is located in Sinaloa State at Northwestern side of Mexico. The plant is divided into four sections: (A) raw milk reception, (B) cheese production, (C) cold storage room and (D) administrative office. A flow diagram of the production of QF with pasteurized milk at the CPP is shown in Fig. 1. The CPP obtains raw milk from two local suppliers, uses stainless steel equipment and utensils, and has six rotary personnel. The CPP has a daily capability of approximately 5,000 L of raw milk that produces 500 kg of QF. The production of this plant is distributed to supermarkets and restaurants throughout the Northwestern region.

Sampling Design

Two visits were made to the CPP in a period of three months. The first visit (I) was performed to demonstrate the hygiene and sanitation of the plant. Samples were collected before operations begin, and after the cleaning and sanitizing processes. The next visit (II) was conducted to collect samples during the cheese-making process. A total of 86 samples were taken from four different categories; food contact surfaces ($n = 44$), hands of workers ($n = 19$), raw milk ($n = 5$), dairy products ($n = 9$) and QF ($n = 9$) (Table 1). All samples were collected following the APHA (2001). Briefly, contact

surfaces and hands samples were collected using a sterile sponge by swabbing an area approximately of 50 cm^2 and 250 cm^2 respectively. The surface samples were taken from the same sites during the sampling periods, but the hands of workers samples were taken depending on their work schedule. For food samples (raw and pasteurized milk, whey, curd and QF), portions of 100 g and/or 100 mL were aseptically placed in a sterile bag. The samples collected were transported in an isothermal container to the National Food Safety Laboratory Research at CIAD for analysis.

Microbiological Analysis

S. aureus was isolated and enumerated using the method described in the Bacteriological Analytical Methods (Bennet and Lancette 2001). Briefly, 10 g or mL of each sample were mixed individually with 90 mL of Peptone Water 0.1% (Dibico, Mexico city, MEX) and homogenized for 2 min; following by 10-fold serial dilutions to 10^{-6}. Each dilution was spread by triplicated onto plate containing Baird Parker (Oxoid, Basingstoke, Hampshire, UK) agar (BP) supplemented with egg yolk tellurite emulsion (Oxoid, Basingstoke, Hampshire, UK) and incubated at 37°C for 48 h.All typical colonies of *S. aureus* (circular black colonies with an opaque zone with an outer clear zone) were enumerated, and polymerase chain reaction (PCR) was conducted in five typical colonies selected from the BP agar to subsequently molecular confirmation. The primers used for PCR-confirmation of *S. aureus* corresponding to thermonuclease gene (F:GCGATTGATGGTGATACGGTT/R:CAAGCCTTGACGAACTAAAGC) with a fragment size of 276-bp (Wang *et al.* 2003). The number of *S. aureus* was reported as logcfu/g, mL or cm^2 of samples tested taking into account the total colonies counted (TC), the number of PCR-confirmed colonies (CC), the selected presumptive colonies (SC), the dilution (D) and inoculated volume (V) onto BP agar. The formula employed was: $\frac{TC \ x \ CC}{SC} x \ D \ x \ V$

PFGE Typing Analysis

To determinate the genetic relatedness of *S. aureus* isolates and the identification of contamination sources at the cheese-processing plant, PFGE typing was performed. DNA from lysed bacterial cells in agarose plugs was prepared according to McDougal *et al.* (2003). Plugs digested with *SmaI* (30 Units) (Promega, Madison, Wisconsin, USA) were run in a Seakem agarose gel (BioRad) with 0.5 X Tris-Borate EDTA buffer (Amresco, Solon, Ohio, USA) on the CHEF Mapper PFGE system (Bio-Rad, Hercules, California, USA). Running parameters were as follows: 200 V (6 V/cm); temperature 14°C; block one: initial switch 2 s; final switch 7 s; for 10 h; and block two: initial switch 8 s; final switch 45 s for 14 h. *Salmonella* Braenderup digested with *XbaI* (Promega, Madison, Wisconsin, USA) was used as the molecular reference marker. Gel images were analyzed by BioNumerics software (version 4.0, Applied Maths) using Dice coefficient and generated by the unweight pair group method with arithmetic mean (UPGMA) with an optimization and a tolerance level of 1%. The isolates that exhibited identical (100% similarity) and related (90% similarity) PFGE profiles were considered to belong to the same clone. The clones were labeled with capital letters *(A, B. C, etc.)* + *Arabic number* for related clones.

Statistical Analysis

A descriptive analysis was performed to determine the levels of contamination and relatedness of bacterial clones using the Statistical Package for the Social Sciences (Statics IBM SPSS version 21).

RESULTS

Detection and Enumeration of *S. aureus* in the CPP

A total of 86 samples of contact surfaces, hands of workers, basic ingredients and QF samples were screened for the presence of *S. aureus* in one CPP located in the Northwestern State of Mexico; from these 18.6% (16/86) samples were positive for the studied pathogen. Table 1 shows the frequency and enumerations of *S. aureus* at the CPP during visits I (after sanitation), and visit II (during cheese production). Visit I was negative for the pathogen tested, reflecting the effectiveness of hygiene and disinfection protocol used when the QF was not produced. In contrast, visit II showed a gradual increase of pathogen prevalence 23.2% (16/69), presumably as a result of the intensive production of QF, the improper handling of personnel and the use of unsafely raw milk, making easer the contamination of the plant environment and the dairy products. Arguing that, *S. aureus* were detected in the 20% (1/5) of raw milk, 18.2% of food contact surfaces (6/33) and 15.4% of worker's hands (2/13). It should be point out the high frequency of *S. aureus* (55.5%) detected in QF samples in one production lot.

The Table 1 indicated the minimal and maximal counts of *S. aureus* detected among the different categories samples tested during visit II. As a general trend, the food contact surfaces and hands tend to maintain a contamination level between 2.0 and 4.3 logcfu, while the dairy food samples (raw milk, whey, curd and QF) showed *S. aureus* levels upper to 3.3 logcfu. These samples categories showed levels over the permissible official limits establish by NOM-243-SSA1-2010 (>3.0 logcfu/g of QF). In this context, 11 (68.8%) of out 16 samples of different categories analyzed could be

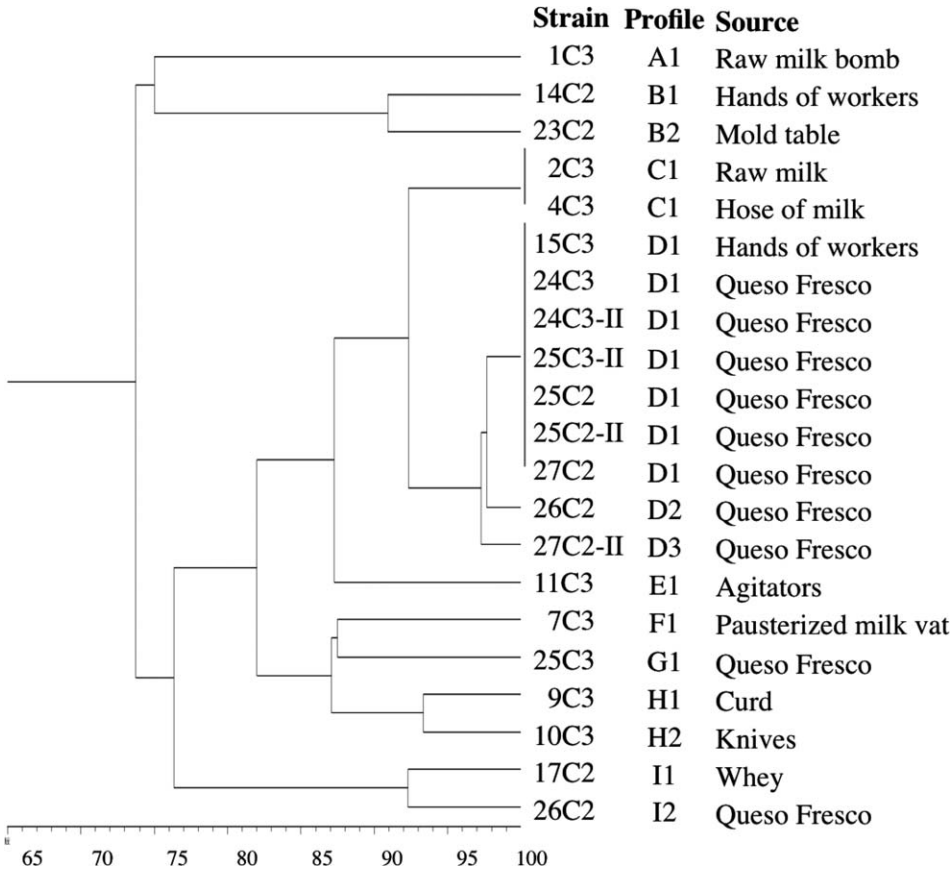

FIG. 2. DENDROGRAM SHOWING *SMAI* RESTRICTION ENZYME PFGE PATTERNS OF GENOMIC DNA OF *S. aureus* ISOLATED FROM THE CPP ENVIRONMENT

considered unsafe in agreement with the norm (NOM-243-SSA1-2010).

Molecular Characterization of *S. aureus* by PFGE

PFGE analysis was performed on *S. aureus* to determine the genetic relatedness between strains recovered from CPP, and to identify possible harboring points of the bacterium. Based on the PFGE genotyping, nine different PFGE patterns (A–I) were observed from the 26 *S. aureus* strains isolated from food contact surfaces, hands of workers, raw milk, dairy products and QF samples (Fig. 2). The D-PFGE patterns represented the predominant clone at CPP, and were shared among nine strains belonged to QF (24C3, 24C3-II, 25C3-II, 25C2, 25C2-II, 27C2, 26C2, 27C2-II) and hands of workers (15C3), this genetic relatedness indicated personnel hygienic failures during cheese making. Likewise, each of the B, C, G and H patterns were represented by two strains belonged from different origin samples, the genotyped similarity observed allowed identify the hands of workers, raw milk, and utensils as a contamination sources, suggesting the possible transference of microorganism throughout these sources to other points within the CPP during cheese manufacturing (Fig. 2). The remaining strains ($n = 4$) of *S. aureus* were typed in a unique PFGE pattern (A, E, F), showing the existence of multiple contamination sources, and a harboring point of the bacterium. Another relevant data, it is the multiple *S. aureus* types contaminated the QF final product samples. For example, isolates from the same QF samples namely 25C3 displayed two different patterns (D1 and G1), which could indicate multiple contamination sources of *S. aureus* in QF. Figure 1, describes the widely distribution and consistency of *S. aureus* clones in the CCP during the cheese making process.

DISCUSSION

Our study is one of the first evidence of the prevalence, levels, and PFGE types of *S. aureus* identified in Mexican QF, showing the potential microbial contamination sources involved in the transfer of the bacterium along of the QF chain production in Mexico (Fig. 1). The results demonstrated that QF harbor *S. aureus*, as a result of improper food hygiene practices that were recorded during production, preservation, and

storage of QF at the CPP (Fig. 1). The data generated are relevant since food poisoning outbreak related to consumption of QF (Arias 2015) or dairy products (Cabrera 2012) have been reported in the region. The identification of the sources and clones of *S. aureus* involved in the final contamination of QF product allowed strengthening the GMHP and designing strategies to control the contamination at the CPP level. The identification of sources harboring *S. aureus* in the CPP of this study is consistent with previously reported by Simeão do Carmo *et al.* (2002) and Kousta *et al.* (2010), suggesting similar conditions in the cheese making, and the risk of obtaining an unsafe product. The strategy for preventing and controlling the spread of microbial contamination along the food chain in a plant is the correct application of GMHP (Kadariya *et al.* 2014). It was observed that the CPP applies GMHP to sanitize the plant environment before the manufacture of QF (visit I), however, as the production moves forward and food handler activity increased, these practices tend to fail along the production, favoring the spread of contamination during the process, and consequently the loss of the safety of processed QF.

The high frequency of *S. aureus* (55.6%) in QF was consistently along its production with a widely distribution of the bacterium in food contact surfaces and raw milk. Taking into consideration that QF could be made with raw milk or the pasteurization could be not enough to maintain the safety of the product, and widely distributed and consumed, the results showed a high public health risk to the consumers. Cases of food intoxication by *S. aurues* are still scarcely reported in Mexico, but in other countries the detection of the bacterial or staphylococcal toxins in cheese or processing environment are related with foodborne cases, making easer the control and prevention of it. For example, in Brazil, in 1999, Carmo *et al.* (2002) described foodborne outbreaks linked to the consumption of Minas cheese and raw milk contaminated with *S. aureus*, affecting a total of 378 individuals. The source of contamination was the hands of workers and the mastitis cattle. Recently, a survey by Lee *et al.* (2012) demonstrated the persistent contamination and widely distribution of *S. aureus* in raw milk 5.5% (12/220), hands of milkers 3.3% (4/120), and equipment and utensils 3.6% (14/389) in a Brazilian milk processing plant. Demonstrating that if proper measures are taken in the plant environment and production the risk of staphylococcus food poisoning outbreaks decreased considerably.

Additionally, Araujó *et al.* (2002) suggested that cheese is an important vehicle of *S. aureus* due to the high frequency 20% (9/45) and high counts that exceeded the limits allowed by the Brazilian's legislation (>3.0 logcfu/g), which has been represented the occurrence of staphylococcal food poisoning outbreaks in Brazil (Pereira *et al.* 1996; Carmo *et al.* 2002). Our study showed a higher incidence (55.5%) of *S. aureus* in QF samples with a levels of >6.5 logcfu/g, which were above of the permissible limit (>3.0 logcfu/g) in 25 g of QF samples establishing by NOM-243-SSA1-2010, implying a health risk to the consumers. It is been suggested that *S. aureus* counts above 5 logcfu/g in food means the association with staphylococcal enterotoxins presence, and its relation with staphylococcal food poisoning (Le Loir *et al.* 2003; Hennekinne *et al.* 2012; Kadariya *et al.* 2014). The present study suggests that QF samples tested may represent a high risk to staphylococcal food poisoning outbreaks, but more studies with high number of samples and the right identification of staphylococcal enterotoxins are needed to make this assumption.

Figure 1 shows the wide distribution of *S. aureus* clones and their relatedness. Tondo *et al.* (2000) applied PFGE to identify multiple microbial harboring points, and highlighted the raw milk as the main source of *S. aureus* in the final dairy products. Aires-de-Sousa *et al.* (2007) demonstrated that raw milk might contribute to *S. aureus* distribution throughout the QF chain. In our study, the raw milk was contaminated with a clone (B1), which was related with the strain observed in milk hose connected with the pasteurizer tank, suggesting also a distribution of *S. aureus* along the CPP production chain. It should be noted that the pasteurized milk was free of *S. aureus* contamination, demonstrating the effectiveness of pasteurization treatment (63°C/30 min) (NOM-243-SSA1-2010), however a post-contamination of QF occurred as it was demonstrated by the presence of different PFGE patterns. Since the PFGE pattern (B) identified in milk was not linked with the personnel of CPP environment, the mastitis cattle could be the main contamination source of *S. aureus*, as previously supported by Simeão do Carmo *et al.* 2002; Kousta *et al.*, 2012; Lee *et al.* 2012.

The *S. aureus* strains identified on the final QF products were genetic related to hands of workers showing the D-PFGE pattern (Fig. 2). Humans are considered the main source of staphylococcal food poisoning because the bacterium is commonly found in the nasal mucous membrane and skin (El-Shenawy *et al.* 2013). The detection of *S. aureus* clone of human origin means a direct hand contact from infected carriers, an intensive handling and lack of hand hygiene. Thus, we suggest tools for training and education the workers, as well as the implementation of intervention strategies at the CPP to avoid the prevalence and distribution of *S. aureus* clones. The fact that a large percentage of QF samples tested positive for *S. aureus* and were linked to food handler contamination means a public health concern because the Mexican population as a cultural habit regularly consumes handcrafted QF, and there is a lack of information on health issues.

The study showed a predominant clonal group in QF samples (Fig. 2), which was persistence in all the samples, favoring its dissemination to the CPP. Also, it was found different *S. aureus* clonal lines involved in the harboring or transferring of this pathogen within the CPP. The high

number of *S. aureus* patterns (A–I) at the CPP could be related to schedule workers, and the incoming new raw material from different milk farmers to the plant, which promote the participation of multiple contamination sources. Additionally, we showed the importance of collecting and analyzing multiple isolates from a single sample, because there may be more than one *S. aureus* subtype and, multiple contamination sources, which can be unrelated. Fagundes *et al.* (2010) and Lee *et al.* (2012) suggested a wild circulation and geographical dissemination of specific *S. aureus* clones by means PFGE analyses, forcing the search for contamination sources and transmission routes to avoid or minimize the occurrence of foodborne diseases. Typing *S. aureus* resulted in a useful tool to track *S. aureus* contamination origin. Into prospective, prevention and control measures must be based on restructured the program of GMHP throughout the food chain to ensure food safety.

CONCLUSION

The source and levels of *S. aureus* found in the present study reveals deficiencies in the cleaning and disinfection processes during pasteurized QF production, mainly on food contact surfaces and hands of workers. The detection of different genetic profiles of *S. aureus* strains suggested multiple contamination sources that reach QF final products. Also, it is been demonstrated that raw milk contaminated by *S. aureus* is related with cow's infections and improvements on better hygiene practices at any CPP are needed to avoid outbreaks. Our results highlighted the use of PFGE to trace bacterial contamination along the food chain, and design appropriate corrective measures to eliminate and prevent microbial harborage points within a food processing plant. The information of the present study should be taken by the competent authorities to enhance surveillance of regulatory sanitary requirements, good hygienic practices and optimization of technological process at any CPP, to ensure the quality and safety of QF.

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DISCLOSURE OF CONFLICT OF INTEREST

No conflict of interest to declare. The authors are responsible for the content and writing of the study. No funding or support of any kind was received from any organization or company.

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