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Full Length Research Paper

Biotype, antibiotype, genotype and toxin gene *tsst-1* in *Staphylococcus aureus* isolated from Cotija cheese in the state of Guerrero, México

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Milk and dairy products such as Cotija cheese are susceptible to contamination by *Staphylococcus aureus*. Some isolates of these bacteria carry the genes for enterotoxins and also the toxic shock syndrome toxin (TSST-1), additionally, many of these strains are potentially resistant to antibiotics. In the present study, a total of 50 samples of Cotija cheese sold at the Central Market "Baltazar R Leyva Mancilla" in the State of Guerrero were collected to determine the amount of bacteria growing in the cheese (expressed as CFU/g of cheese), the biotype, the antibiotype and the genotype. The results show that the amount of bacteria varies from 12×10^3 to 3×10^6 CFU/g exceeding the permitted limits for ripened cheeses. Three biotypes were identified, 100% were susceptible to oxacylin, vancomycin, ciprofloxacin, trimethoprim-methoxazole, amikacin and clindamycin but tetracycline resistant. Only one of the four identified genotypes was positive to the TSST-1 gene. This is the first report of *S. aureus* isolated from Cotija cheese in the state of Guerrero, Mexico. Finally, our data evidenced cotija cheese as a vehicle carrying a large number of pathogenic *S. aureus* strains, suggesting that the public policy on food safety must be firmly reviewed.

Key words: *Staphylococcus aureus*, biotype, antibiotype, genotype, *tsst-1*, cheese.

INTRODUCTION

Staphylococcus aureus is a pathogenic bacterium in humans and animals, capable of producing a variety of toxins, as the enterotoxins A, B, C, D, E, G, H, I and J (also known as SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ, respectively) (Sospedra et al., 2012;

Gouloumes et al., 1996; Morandiet al., 2007). It also produces the toxic shock syndrome toxin 1 (TSST-1) which has a variety of effects on the host immune cells (Chapaval et al., 2006). As it is able to act as a super antigen, it leads to a massive release of cytokines

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including tumor necrosis factor alpha (TNF- alpha), interleukin 1 (IL -1) and IL- 6, thus causing capillary leak syndrome responsible of the TSST-1 symptoms (Sospedra et al., 2012). *S. aureus* has been isolated from clinical and environmental samples, in healthy carriers (nasal cavity, hands, etc.) and food. Symptoms associated with the consumption of food contaminated with *S. aureus* carrying the TSST-1 gene, usually present diarrhea, abdominal pain, cramps, exhaustion and in more severe cases include headache, muscle cramps, fever, vomiting, confusion, conjunctival hyperemia, hypotension, oliguria, renal damage, changes in blood pressure and pulse (Soriano et al., 2002; Sospedra et al., 2012), appearing from 1 to 6 h after ingesting contaminated food.

The probability of food poisoning by *S. aureus* (Necidova et al., 2009), is given by either the presence of only one of the enterotoxins or a microbial amount equal to or greater than 10^5 CFU/g (Kerouanton et al., 2007). Strains of *S. aureus* capable of producing enterotoxins isolated from cows, sheep, buffalo and other dairy products have been reported in Italy (Morandi et al., 2007). There have also been reports of outbreaks associated with the consumption of food in restaurants, buffets and cafeterias in USA, Japan, United Kingdom, Thailand, France, England and Mexico (Soriano et al., 2002). In France, *S. aureus* is the second microorganism associated with food borne infections after *Salmonella* sp. (Kerouanton et al., 2007). In the state of Guerrero, the Ministry of Health reported an outbreak caused by *S. aureus* and *Salmonella* sp. that led to 335 poisoned people, including 317 children and 18 adults. This happened during the celebration of the Children's Day at an elementary school in the village of Organs (<http://prosaia.org>). We were interested since it is of interest to study the food sources that may be contaminated with the bacteria. The strains of *S. aureus* producing the TSST-1 toxin isolated from food have been also associated with antibiotic resistance. Among the known cases are the isolates resistant to methicillin (MRSA) which confers resistance to all beta-lactamase antibiotics, making the treatment ineffective and being the cause of hospital outbreaks (Montesinos et al., 2002). The high health risks associated with MRSA infections in hospitals and in the community have prompted the government to implement surveillance and monitoring programs. It is important to note that in the city of Chilpancingo, Gro., Mexico, Cotija Cheese constitute an important part of the dairy diet. The Cotija cheese is a Mexican handmade product with unique features in the world, which is derived from the coagulation of milk proteins, mainly casein, followed by a draining, salting and molding. The process of making cheese is completely handmade and begins with the milking of the cow to obtain milk then coagulant agent is added. This coagulation process removes water and the greater amount of lactose. Then, the required amount of salt is manually added. At least three months of maturing is

required to ensure their sensorial and microbiological quality. Due to the existence of critical points or cross-contamination by the lack of good hygienic practices, Cotija cheese constitutes an important substrate for *S. aureus* growth. When temperature is above 40°C (normal room temperature in Guerrero), the genes for enterotoxins and toxic shock syndrome are activated in *S. aureus*. The aim of this study was to quantify the microbial load of *S. aureus* isolates in the Cotija cheese sold at the central market in the city of Chilpancingo, Guerrero, as well as to determine the phenotype and genotype of the isolated strains. The isolates were analyzed for biotype antibiotic resistance profiles and by PCR for the presence of TSST-1 gene. The genotype was determined by restriction pattern PFGE profile. This allowed us to compare and conclude the genetic relationship among our isolates and previously collected samples of Cotija cheese that were contaminated too.

MATERIALS AND METHODS

Food samples collection and processing

A total of 50 samples of Cotija cheese (250 g each) were collected from different spots at the central market Baltazar R Leyva Mancilla in the city of Chilpancingo. They were collected from August to October 2012 following the recommendations stated by NOM -109-SSA1-1994, which refers to the Decision Procedures for Handling and Transportation of Food Samples for Microbiological Analysis. All samples were analyzed at the Laboratory of Microbiology Research of Food and Beverages at the Autonomous University of Guerrero.

Isolation and identification of *S. aureus*

Isolation and identification of *S. aureus* from Cotija cheese samples were performed by using the guidelines of the NOM-115-SSA1-1994, specifying the Goods and Services Method for the determination of *S. aureus* in food. Colony forming units (CFU/g) were calculated per gram of Cotija cheese from each of the samples in the same way it was done previously for a common standard brand.

S. aureus was grown in Baird Parker agar with egg yolk emulsion potassium tellurite. 1% agar plates were incubated at 35°C for either 45 or 48 h. After this time, typical black, round, shiny, convex, smooth, diameter 1-2 mm colonies were observed. They showed opaque zones and clear halos surrounding the colonies. The biotype of the *S. aureus* isolates was confirmed by conventional biochemical tests that included: growth on Mannitol Salt Agar, Gram staining (where Gram positive cocci grouped in clusters were observed), the production of specific enzymes including coagulase, catalase, thermonuclease lecithinase and finally, the ability of fermenting carbohydrates such as glucose, mannitol, lactose, sucrose, maltose and trehalose.

DNA extraction

The DNA from the *S. aureus* isolates was extracted as previously described by Morandi et al. (2007).

Table 1. Phenotypic characteristics of *S. aureus* in Cotija cheese sold at the market of Chilpancingo city, Guerrero, Mexico.

Biotype	Lec	Term	No. (%) of isolates	SyM	Carbohydrates				
					Glu	Lac	Sac	Mal	Tre
1	-	-	8 (23)	+	+	+	+	+	+
2	+	-	18(51)	+	+	+	+	+	+
3	+	+	9 (26)	+	+	+	+	+	+

Lec: lecithine, Term: termonuclease, SyM: mannitol salt agar, Glu: glucose, Lac: lactose, Sac: sacarose, Mal: maltose and Tre: Trehalose.

PCR detection of the *tsst-1* gene

Purified DNA was used for PCR detection of the TSST-1 gene using the following oligonucleotides; GTSSTR -1 5'-ACCCCTGTTCCCTTATCATC -3' and GTSSTR -2, 5'-TTTTTCAGTATTTGTAACGCC -3' (Chapaval et al., 2006) which amplify a product of approximately 326 bp. PCR was performed with a 50 µl reaction mixture containing 1 µl (10 ng) of DNA as template, each primer at a concentration of 5 µM, 2.5 mM MgCl₂ and dNTP's at a concentration of 2.5 µM, as well as, 2 U of Taq DNA polymerase (Platinum® Taq DNA polymerase Invitrogen, Carlsbad, CA, USA) using the amplification protocol described by Chapaval et al. (2006). DNA of *S. aureus* ATCC 25923 was used as negative control in each round of PCR. All PCR reactions were carried out in a Mastercycler gradient Cycler® Ep (Eppendorf, Hamburg, Germany). PCR products were visualized on 2% agarose gels stained with ethidium bromide and visualized in the UV transilluminator (Bio-Rad system).

Antibiotic resistance assay

Both, the antibiotic resistance assay and the bacterial identification were performed by Post Combo Panel Type 41 and read on a WalkAway System (Dade Bering Inc., West Sacramento, Calif.). An inoculation control (0.5 McFarland units) was used for all the assays. The antibiotics tested were oxacylin (OXA), tetracyclin (TET), chloramphenicol (CHL), clindamycin (CLI), trimethoprim-sulfamethoxazole (SXT), gentamicyn (GEN), erythromycin (ERY), fosfomicyn (FOF), amikacin (AMK), rifampicin (RIF), ciprofloxacin (CIP), vancomycin (VAN) and teicoplanin (TEC). Bacterial isolates growing in at least one different antibiotic from the rest was considered to belong to a different antibiotic type.

Pulsed field gel electrophoresis (PFGE)

Molecular typing of the whole genomic DNA was done as previously described (Chung et al., 2000). After digestion with *Sma*I endonuclease, DNA was separated in a CHEF-DRII apparatus (Bio-Rad, Birmingham, UK) (Chung et al., 2000). We used the Tenover criteria for the interpretation of the PFGE patterns were the profiles were compared by visual inspection (Tenover et al., 1995).

RESULTS

Identification of *S. aureus* in the Cotija cheese samples

Fifty four percent (27 out of 50) of the collected samples

was found to be positive for *S. aureus*. The morphology of the colonies grown on Baird Parker agar plates with egg emulsion and potassium tellurite 1%, was typical for *S. aureus*. The colonies were black, circular, shiny, convex, smooth and had diameters from 1 to 2 mm. They showed opaque areas and clear halos surrounding the colonies. From the 27 samples that resulted positive, 35 different isolates of *S. aureus* were obtained, 77% of these (27 out of 35) were found to be lecithinase positive.

S. aureus counts in the 27 contaminated samples increased from 12x10³ to 3x10⁶ CFU/g. The maximum allowable limit (CFU/g) of *S. aureus* in mature cheese (such as Cotija) is 100 CFU/ g as specified by NOM-121-SSA1-1994 (Goods and Services Cheese; Fresh, ripened and processed sanitary specifications). So, all the cheese samples analyzed exceeded the maximum permissible limits.

Three *S. aureus* biotypes identified

The biochemical characteristics of the *S. aureus* isolates are as follows: Biotype 1 representing 22.85% of the isolates (8 out of 35) was negative to lecithinase and thermonuclease but positive to coagulase and catalase (data no show). This biotype was found to ferment manitol, glucose, lactose, sucrose, maltose and trehalose. Biotype 2, which represents the largest number of isolates, covering 51.42% (18 out of 35), included isolates positives to the lecithinase assay and negative to the thermonuclease one or vice versa. The rest of the tests were positive in this group. Biotype number 3 includes 25.71% of the samples (10 out of 35), positive phenotypes were observed for all the tests included in the study (Table 1).

S. aureus growing in the Cotija cheese samples was resistant to antibiotics

S. aureus was resistant to four different antibiotics when tried on a panel of 12 different antibiotics (Table 2). 43% of the isolates were resistant to ERY, TET, CHL and RIF, this group was named as Ant 2. 28% was resistant to

Table 2. Antibiotic susceptibility of isolates in this study.

Antibiotype	Susceptibility to:													No. (%) of isolates
	ERY	FOF	GEN	TET	SXT	AMK	CLI	CHL	RIF	CIP	VAN	TEC	OXA	
Ant1	R	S	S	R	S	S	S	R	R	S	S	S	S	8 (23)
Ant2	R	R	R	R	S	S	S	S	S	S	S	S	S	15 (43)
Ant3	S	S	S	R	S	S	S	S	S	S	S	S	S	10(28)
Ant4	S	S	R	R	S	S	S	R	R	S	S	S	S	2 (6)

R, resistant; S, susceptible. ERY, erythromycin; FOF, fosfomicin; GEN, gentamicin; STX, trimethoprim-sulfamethoxazole; AMK, amikacin; CLI, clindamycin; CHL, chloramphenicol; RIF, rifampin; CIP, ciprofloxacin; VAN, vancomycin; TEC, teicoplanin; OXA, oxacylin.

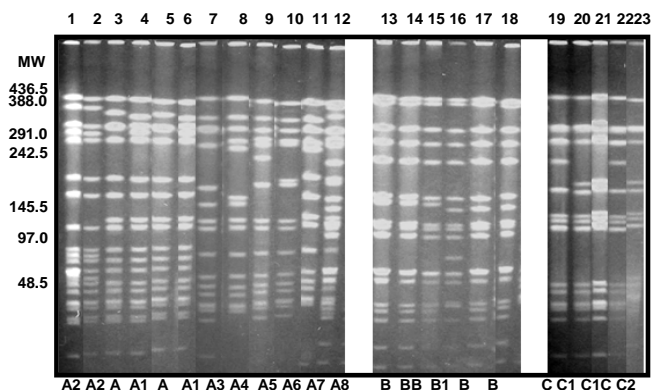


Figure 1. Examples of pulsed-field gel electrophoresis profiles obtained for *S. aureus* isolates from the Cotija Cheese, Guerrero, Mexico. Lanes: 1, lambda ladder used as molecular size (MW) marker; 2 to 23, isolates in this study.

TET (Ant 3) (Table 2). All the isolates were sensitive to OXA, VAN, TEC, STX, AMK, CLI and CIP.

The TSST-1 gene was present in one of the isolates

As for PCR detection of the TSST-1 gene, only the M9 strain proved to be positive for the presence of the TSST-1 gene that represents 2.85% of the total collection (data not show).

Genome profiles of the isolates are different

Twenty three isolates were classified in three PFGE patterns. Pattern A include 8 subtypes (A1 to A8), pattern B only one subtype (B1) and finally the profile C had two subtypes (C1 and C2). The patterns were different in up to six bands (Figure 1).

DISCUSSION

The dominant populations of microorganisms in the Cotija cheese are lactic acid bacteria that help to maintain shelf life for extended periods of time, preventing the growth of pathogenic microorganisms capable of causing disease (Campos et al., 2008). The contamination of food by *S.*

aureus is given by strains that have and express genes for enterotoxins and/or the toxic shock syndrome toxin 1 (Gouloumes et al., 1996). Coupled with the presence and expression of genes encoding the enzymes described above, the susceptibility to antibiotics has been associated with strains of *S. aureus* isolated from clinical and food samples. One of the sources of contamination causing infection by strains producing enterotoxins and TSST-1 is precisely contaminated food (Sospedra et al., 2012), among which are the dairy products, such as cheese which has a high risk of bacterial contamination. Cotija cheese is sold in creameries Market Baltazar R Leyva Mancilla in the city of Chilpancingo, most exceed the permissible limits of the bacterial load of 12×10^3 to 3×10^6 CFU/g, given that, the maximum allowable limit (CFU/g) of *S. aureus* in mature cheese is 100 CFU/g as specified in NOM-121- SSA1- 1994. These data differ in some cases with those reported by Okinenden et al. (2008), a study carried out in Germany where they worked on Curd (cream) cheese, soft cheese, semi-hard cheese and hard cheese prepared from pasteurized raw milk and in all cases the bacterial load ranged between 3×10^1 - 3.1×10^3 , 5.3×10^1 to 1.3×10^5 , 8×10^1 to 5×10^1 to 3×10^4 and 8.6×10^4 , respectively.

Cotija cheese contamination can be due to many factors which include manipulation while obtaining milk, no milk pasteurization, inadequate hygiene of milk handling, contamination of containers where the product is prepared, storage of cheese during the ripening process in the stand and transport chain from retail outlets to the consumer. All this can lead to contamination of the product, which is why good hygiene practices are important for decreasing *S. aureus* contamination in food as described by Soriano et al. (2002). When the bacteria reach the product, they are able to multiply and cause infection in people who consume them, coupled with contamination by pathogenic bacteria, the ability to produce bacteria toxins, which include enterotoxins A, B, C, D and E, and the toxic shock syndrome toxin 1. In this study, we determine the microbial load in all the samples collected and it is the first report of *S. aureus* growing in Cotija cheese in the State of Guerrero. The results shown are alarming because the Cotija cheese is a staple in Guerrero and may cause a public health problem. The three biotypes highlight

toxin production as lecithinase and termocucleasa which turns them into a more pathogenic *S. aureus*. 100% of the strains were sensitive to oxacylin, vancomycin, teicoplanin, ciprofloxacin, amikacin, these findings is consistent with that of Tsen et al. (1998) which reported sensitivity to penicillin, oxacylin, vancomycin and others. We detected the gene encoding the toxin (TSST-1) in 2.8% of the samples (only strain M9), these data is consistent with Sospedra et al. (2012), where 0.1% (1/53) of the isolates collected from food handlers and food service establishments in Spain was shown to have the gene. Furthermore, around the world, Oh et al. (2007), detected TSST-1 in 13.5% of food samples in Korea. Rapine et al. (2005) found that 4.4% of the *S. aureus* isolates from goat's cheese handlers produced TSST-1. In another study conducted by Abdulmula El-Ghodban et al. (2006), the TSST-1 gene was detected by PCR in three strains (all from clinical sources). Zschock et al. (2000) detected it in 19 strains of *S. aureus* isolated from bovine mastitis in one case in Germany. All studies show the high frequency of *S. aureus* in foods derived from dairy products, clinical samples and mastitis carriers with the capacity to express TSST-1. Our results demonstrate the genotype of three clones of *S. aureus* isolated from Cotija cheese matches the antibiotype and the biotype so our findings are consistent with that of Peles et al. (2007) which showed genetic relation between the *S. aureus* strains recovered from quarter milk and bulk milk in two large farms, implying that farms have a high number of mastitic cows, etc.

In conclusion, we reported three clones (based on their biotype, antibiotyping and genotype) of *S. aureus* isolated from Cotija cheese from the central market in the city of Chilpancingo, Guerrero, Mexico. All samples exceeded the maximum permitted levels for human consumption in mature cheese, thus representing an important source and a risk factor for food borne infection which represents a serious public health problem in the exposed population. It should be noticed that just the M9 strain has the TSST-1 gene which encodes for the toxic shock syndrome toxin, so it is the first case reported in Mexico. All strains were sensitive to oxacylin and vancomycin which is noteworthy because although this bacterium is present and exceeds the maximum, it may be limited by antibiotics. Further studies are needed to determine the presence of genes coding for enterotoxins A, B, C, D and E in *S. aureus* isolated from foods in the State of Guerrero and recommend sanitation measures employed in the processing of cheese and food in general.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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