

***Staphylococcus aureus* MRSA-biofilms positive isolated from artisanal cheese in Guerrero., Mexico**

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Original article

Abstract

The collection, preparation and processing of lactic products made from raw cow's milk can lead to contamination with *S. aureus* strains. Contaminated artisan fresh cheeses with *S. aureus*, can cause outbreaks in people who consume tis products. **Aim.** determine the presence of *S. aureus* MRSA strains and biofilms positive in fresh artisan cheese distributed in Chilpancingo, Gro. **Material and Methods.** We determined the microbial load, identified, evaluated resistance to methicillin and biofilm formation in 50 samples of cheese. **Results.** 72% of *S. aureus* samples isolated with 1×10^3 to 1×10^5 CFU / g beyond the maximum allowable limits, were grouped into four biotypes which were positive for biofilms, only 18% were MRSA. **Conclusion.** Fresh artisanal cheeses are contaminated with MRSA strains, it requires specialized epidemiological surveillance and propose good hygienic practices in the development of cheese to prevent possible outbreaks of *S. aureus* by milk products.

Keywords: artisan cheese, *S. aureus*-MRSA, biofilms positives, biotypes, outbreaks.

Introduction

The cheeses are processed products of the cow's pasteurized milk curdles. The artisan cheese is made from raw milk, usually cows Creole, with spontaneous fermentation and short maturation using very rudimentary methods not standardized in where the majority are traditional and artisanal products that are manufactured in small batches with particular attention to the traditional art of the

cheese, using the least number of mechanical processes in the production of the same. This creates the possibility of sources or risk factors for cross-contamination occurs.^{1,2}

Due to its poor quality hygienic and microbiological can be an ideal vehicle for the growth of various microorganisms mainly bacterial strains more frequent in the artisan cheeses correspond to the contamination with strains of *S. aureus* and *Salmonella* sp, total and fecal coliforms (*Escherichia coli*, *Enterococcus faecalis*) as well as the presence of *Lactobacillus* sp.¹ *S. aureus* stands out as the main contaminant in dairy products including fresh cheese and is the agent microbiological with greater frequency is related to food poisoning by the consumption of food that contains sufficient amounts of one or more enterotoxins that this bacterium produces.

In the year 1993 to 2000 in Venezuela 48 sprouts were described estafilocócicos where 40 of them involve to the cheese,³ in Latin America (1993-2002), 719 sprouts were detected estafilocócos affecting 27 693 persons of whom³ died,⁴ in Mexico the National Laboratory of Public health realized a poisonings review for food in 1980 and 1989, in that they bring to *S. aureus* like the main problem with 48.2 % of the sprouts,⁵ in Venezuela described a load microbial in white cheeses he was to 103-104 UFC/g in 40 % of specimen.⁶

S. aureus is a pathogen capable of causing various diseases by the production of enterotoxinas,³ the biofilm formation of this bacterium leads to the persistence of the pathogen in the environment, forming microbial communities structured, which entails adherence to various surfaces, the accumulation and maturation of biofilms, by what this phenomenon is described as a factor of virulencia,⁷ coupled with this infections are compounded by the increase of the resistance to antibiotics.³ Antibiotics β -lactam antibiotics are the most used in the treatment of staph infections and due to the increase in methicillin resistance makes *S. aureus* even more formidable, have been described strains MRSA (*S. aureus* resistant to methicillin), around the world in dairy products distributed in markets of Egipto,⁷ by which has been associated as the main etiological agent of foodborne illness present in products dairy.¹

In a study carried out by the same working group in cheeses Cotija is reported the microbial load greater than the maximum allowable limits by the official Mexican norm corresponding, all strains were susceptible to methicillin, and are grouped in different genotypes,^{3, 8} and therefore the aim of the present research focused on determining the microbial load and identify to *S. aureus*, the methicillin-resistance and the ability to form biofilms in fresh artisan cheeses distributed at sales posts within and outside the market Baltazar R Leyva blemish on the City of Chilpancingo, Guerrero, Mexico., the present research was conducted with the purpose to identify and to determine the microbial load and the epidemiology of *S. aureus* in fresh artisan cheeses for sufficient elements useful for proposing

epidemiological control measures to reduce the risk by its dissemination among the consumers.

Materials and methods

Taking of sample, collection and processing: was a descriptive observational transverse character study which was carried out collecting a total of 50 samples of fresh cheeses made of traditional way of selling in fixed positions inside and outside the market Baltazar R. Leyva Mancilla in the city of Chilpancingo, Guerrero, Mexico. These were collected from August to October 2013 following the recommendations of the NOM-109-SSA1-1994. All samples were transported to the laboratory of Microbiology of foods and beverages of the Autonomous University of Guerrero.

Isolation and identification of *S. aureus*: from each sample of fresh cheese is made by following the recommendations and procedures of the NOM-121-SSA1-1994, for the processing of fresh cheeses, ripened and processed, as well as the recommendations of the NOM-115-SSA1-1994, goods and services. Methods for the determination of *S. aureus* in food. The colony-forming units (CFU/g) were calculated per gram of fresh artisan cheeses.

All samples were inoculated on Baird-Parker Agar with egg yolk emulsion more potassium tellurite to 1%. Subsequently incubated at 35 ° C for 45 to 48 hours, after this time were observed and selected typical, round, convex, 1-2 mm diameter black colonies respectively. The selected colonies showed clear halos around the colonies and opaque areas. Biotypes of the *S. aureus* strains were confirmed by conventional biochemical tests which included; growth on agar mannitol, Gram stain, production of catalase, coagulase, lecithinase, termocucleasa and finally the ability to ferment carbohydrates; glucose, sucrose, mannitol, lactose, Xylose, maltose, and trehalose ³ and from the observed biochemical profiles strains were classified into biotypes.

Formation of biofilms: be quantified the capacity of each strain of *S. aureus* biofilm formation using the Crystal Violet test. From a 1×10^8 cfu/ml bacterial suspension, 200 μ L suspension mixed with 1.8 mL of nutrient broth. Later 200 μ L of the prior suspension, were taken and placed in micro-titulation plates and incubated at 35° C for 12 h. Sterility, negative and positive controls were used. After the incubation period the culture medium was removed from delicate way and they were three washes with 200 μ L of PBS buffer pH 7.2 and 9 was quantified. Antibiotics resistance test: determination of resistance to Methicillin in *S. aureus* strains isolated from cheese was made as recommended by CLSI (Clinical and Laboratory Standards Institute, 2013) 10, Mueller-Hinton Agar Disc diffusion method, using disks with a concentration of oxacillin (1.0 μ g), included the disc of Vancomycin.

Results

We analyzed 50 samples of fresh artisan cheeses for sale in retail outlets within and outside the market Baltazar R Leyva Mancilla, of which only 72% (36/50) were positive for the presence of *S. aureus* with a count of CFU/g in each sample that ranged from 1×10^5 to 2×10^3 [Table 1]

Table 1. Detection and identification of *Staphylococcus aureus* from 50 samples of fresh artisan cheeses from Chilpancingo, Guerrero, Mexico.

<i>Staphylococcus aureus</i>	Frequency	%
Positive	36/50	72
Negative	14/50	28

Source; Direct analysis in the laboratory of biotechnology and microbial genetics, UAgro. 2013 [the bioburden was between 1×10^5 to 2×10^3].

Were isolated 61 strains characteristics of *S. aureus*, found their identification with the coagulase test, catalase and more biochemical, only 51% (31/61) were shown to be lecitinasa positive. On the basis of the identification biochemistry strains were grouped into four biotypes, as shown in [Table 2].

Table 2. Biochemical classification by biotypes of strains of *Staphylococcus aureus* isolated from artisanal cheese from sale within and outside of market Baltazar Leyva Mancilla., of the city of Chilpancingo, Gro.

Biotye	Lecit.	Term.	No. (%) of insulation	Man.	Carbohydrate				
					Gluc.	Lac.	Sac.	Malt.	Treh.
1	-	-	41(25/61)	+	+	+	+	+	+
2	+	-	41(25/61)	+	+	+	+	+	+
3	-	+	8(5/61)	+	+	+	+	+	+
4	+	+	10(6/61)	+	+	+	+	+	+

Source; Direct analysis of microbial genetics and biotechnology laboratory, 2013. UAgro [Lecit: Lecithinase, Term: Termonucleasa, Man: mannitol, Gluc: glucose, Lac: lactose, Sac: sucrose, Malt: maltose, Treh: trehalose.] [100% biofilm-producing].

All strains were able to produce biofilms with absorbance at 590 nm of 1,115 to 2,389 , which confirms the ability to persist in the environment where they are processed. The detection of strains MRSA were distributed as follows; biotype 1, 20% (5/25), biotype 2, 16% (4/25), biotype 3, 20% (1/5) and biotype 4, with 17% (1/10), and all were susceptible to vancomycin [Table 3].

Table 3. Detection and distribution of biotypes of *Staphylococcus aureus* MRSA from fresh artisan cheeses in Chilpancingo Guerrero.

Biotype	Frequency	%
1	5/25	20
2	4/25	16
3	1/5	20
4	1/10	17

Source; Direct analysis in the laboratory of biotechnology and microbial genetics, UAgro. 2013 [all strains were sensitive to Vancomycin].

The 18% of the strains of *Staphylococcus aureus* were MRSA, while 72% was sensitive to vancomycin (Table 4).

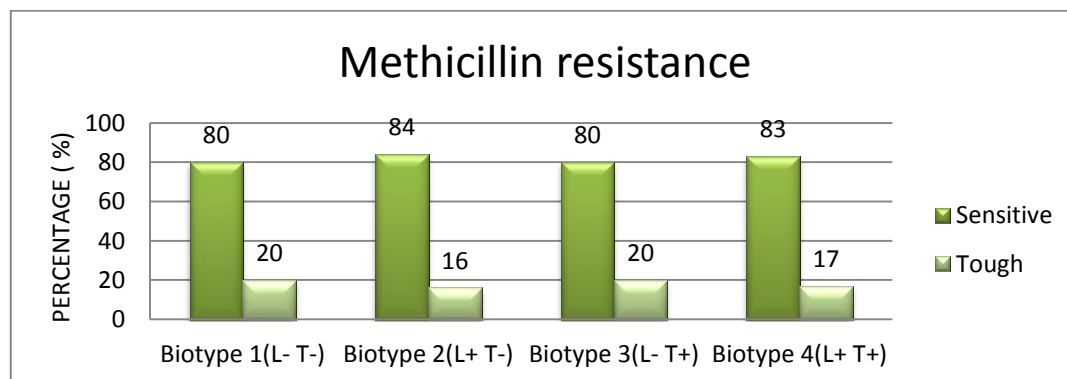
Table 4. Percentage of resistance of *Staphylococcus aureus* from fresh artisan cheeses in Chilpancingo Guerrero.

<i>Staphylococcus aureus</i>	Frecuencia	porcentaje
Methicillin resistance	11/61	18%
Sensitive to Methicillin.	50/61	82%

Source; Direct analysis in the laboratory of biotechnology and microbial genetics, UAgro. 2013

This can also be seen in the classification of strains in 4 biotypes (Figure 1).

Figure 1. Frequency of 4 obtained biotypes Methicillin resistant and sensitive strains.



Source; Direct analysis in the laboratory of biotechnology and microbial genetics, UAgro. 2013

Discussion

S. aureus strains isolated from products dairy products, mainly cheese and within these the fresh prepared using traditional methods are susceptible easy to pollute at any point in the production chain, whether from obtaining milk by infections in the udders of cows (mastitis), the hands of which milk, by people who handled the product vessels where he is produced, cross-contamination by healthy carriers, transport, storage can lead to the contamination of the product and the terms of adaptability of the bacterium is easily its colonization and multiplication, pollution is related to environmental conditions, the presence of salt and the source of carbon in the product. Therefore all these factors lead to cross-contamination of the product with *S. aureus* strains.¹¹

It is important to point out that in the State of Guerrero due to environmental conditions, with regard to the climate and social development status is considered necessary are carried out research projects that allow not only the detection, but the design of strategies to find the food quality control and that in turn, directionally orient health authorities to maintain active and specialized epidemiological surveillance, since in particular the contamination of food, as in the case of fresh artisan cheeses put at risk human health, in this same way the publication of data on the bacterial load and the presence of strains of *S. aureus*, is necessary so that the total of samples obtained in a short period of time he was 72% (36/50) of positive for the presence of *S. aureus* in cheese obtained in the fixed stalls of the market Baltazar R. Leyva Mancilla in the CD. In Chilpancingo, all samples were analyzed and presented a microbial load of 1×10^3 to 1×10^5 cfu/g of cheese, these results exceed the maximum permissible limit described in the NOM-121-SSA1-1994.²²

The results obtained in this research for this bacterium, are similar to those reported by Lemus Espinoza, et to the. (2008) where reported a frequency of the 80.5% of isolates of *S. aureus* in cheese fresh in the street markets of Chilpancingo Guerrero.¹²

Toribio-Jimenez, et to the. (2014), isolated and they quantified the bioburden in Cotija cheese, issued in the same place that fresh artisan cheeses here evaluated, reporting that bacterial in the Cotija cheese charge exceed the maximum limits allowed by the rules mexicanas.¹³ In studies carried out on a farm where they produce small scale production of raw milk containers are collected where milk analyzed where it is processed, they took samples of milk handlers and other more, reporting the contamination in 10 cows by strains of *S. aureus*¹⁴, 75% of the strains were of the same genotype by what is shown in the entire estate is contaminated by this pathogen, this represents a public health problem so raw-milk products are highly susceptible to contamination by *S. aureus*.¹⁵

As to the phenotypic characterization of the *S. aureus* in this work were grouped into four biotypes this might suggest that the origin can be by you clones different, probably the clonality may be associated with the place or staff that

handled, transported or sold the product. Is therefore important to propose further studies to determine the clonal strains origin, given that they are resistant to Methicillin, this data is interesting and important because it may be a predisposing factor for the spread of strains in the community and cause outbreaks and food poisoning, there are strains of *S. aureus* able to express the genes that code for different enterotoxins which include A, B, C, D, and E², as well as the presence of the toxin of the shock syndrome toxic (TSST-1), Ortega, et to the. (2010), reported the discovery of 53 *S. aureus* strains isolated in the hands of manipulators for surfaces of food establishments and food in Spain, they make the first report of tsst-1 in the hands of a worker at the restaurant, demonstrating cross-contamination between handlers and food, the study reported by Toribio-Jimenez, 16 et to the. (2014) in strains isolated from Cotija cheese reported biotypes were found to be sensitive to Methicillin, but only one was able to amplify the gene that encodes for tsst-1, has complicated standardize the multiple PCR in these strains for the presence of genes that encode for described enterotoxins, to look for them in strains isolated from artisan cheese by the epidemiological importance it should be noted that there are other reports on the presence of this bacterium in other dairy products^{2, 13, 17}.

Highlighting the four biotypes found in fresh artisan cheeses and according to what was reported by Manjarrez-Lopez, et to the. (2012) is likely that the frequency of each present in studied cheese biotype is due to inadequate practices in the management of the preparation of the product, which encourages pollution cross between producers and consumers, thus contributing to the spread of bacteria between the susceptible population.¹⁸

It is important to highlight capacity presenting strains obtained from the artisanal cheese produce biofilms, given that is a virulence factor, and leads to the persistence of this bacterium in the environment, this match Lee, et to the. (2014), where they demonstrate the ability of *S. aureus* isolated farms dairy in São Paulo, Brazil, of cows with and without mastitis, of surfaces of machine milking into containers of milk, among others, reported that 45 percent are able to form biofilms.¹⁴

Another study described by Tang, et to the. (2013), reported that *S. aureus* strains isolated from different sources not all have the ability to produce biofilms, and also describes that this capacity can lead to nosocomial infections and cross-contamination of foods for people who consume them,¹⁹ so, it is important to determine this ability in all isolates of *S. aureus*, given that the biofilms confers antibiotic resistance, aggravating the situation of control of microorganisms. The results obtained allow to establish strong differences since the analysis allows to glimpse that it was detected 18% (11/61) MRSA, these data differ with Lemus Espinoza, et to the. (2008), they reported a 32.2%, Pereira, et to the. (2009) 38%, Alvarado, et to the. (2011) by 15% and Kamal, et to the. (2013) reported the prevalence of strains resistant to Methicillin in coagulase negative in dairy products on sale at a market in the province,^{1,12,20,21} derivative of the results obtained with respect to microbial load, as well as its resistance to Vancomycin and metilcilina profiles, as well as other virulence factors associated with the manifestation of risks to public health is strong to attend the following proposal:

Perform scientific research projects based on the methodology from the MRSA strains isolated from products molecular epidemiology dairy for designing control strategies, intervention by the authorities responsible for monitoring the quality of the food, in particular of artisanal, same fresh cheese which represents an area of opportunity for the survival of people engaged in the trade of such products and requiring among other things , from the health education training courses, situation that would decrease the risk to the consumer population, at the same time this would impact positively on the decrease of incidence prevalence of disease outbreaks and by the increase in morbidity and mortality resulting from the distribution or dissemination, since the presence of resistance to Methicillin-Vancomycin as virulence factors , among others might hamper the treatment in case of intoxication by food in the population therefore it is important to consider the application of NOM-121-SSA1-1994²².

As already pointed out Vazquez (2005), *S. aureus* (MRSA) meticolinorresistente is recognized as one of the most important pathogenic causes of nosocomial infections worldwide; the emergence and spread of increasingly virulent and multidrug-resistant strains makes it necessary to make a review of the issue.²³

The central element of resistance to Methicillin in *S. aureus* is the *mecA* gene acquisition, which is endogenous of this bacterium and is integrated into the chromosome., *mecA* encodes for a binding protein (PBP) penicillin 78KDa (PBP2A), which has low affinity for β -lactam antibiotics. A study conducted in 1996 using prototype strains isolated in different continents, provided the first evidence of the existence of three types of chromosomal cassette estafilococicos (SCCmec I-III).^{24,25} Recently, described a fourth type (SCCmec IV).^{26,27} Recent studies indicate that types II and IV are circulating strains of MRSA in Mexico.²⁸ Calderón et al., (2002), in its results already stated that: the resistance of *S. aureus* was 14.2% and 53.4% in the *Staphylococcus coagulase negative Staphylococcus*. Concludes by stating that it is necessary to continuously monitor the progression of resistance of *Staphylococcus* spp. to the meticolina.²⁹ apparently these results are similar to those obtained in the present study, this could mean that *S. aureus* is still being an important micro-organism that must continue to monitor both outbreaks by food, as in nosocomial infections, and required the application of surveillance epidemiologic of foods, especially those that are sold in favorable environmental conditions for growth and maintenance of this type of bacteria it is the sale of artisan cheeses.²⁹

Shedding light on the factors that contribute to the epidemic superiority of clones them of MRSA pandemic, high levels of expression of certain genes for virulence and ability to survive in the environment can be of great importance for the control of MRSA currently circulating and to avoid the emergence of strains with a greater degree of resistance and pathogenicity,²³ and this impacts negatively on the costo-atencion medical for affected people as well as the cost of health institutions, without ignore its impact in style and quality of life not only in the State of Guerrero, but with impact in public health globally.

Conclusion

This research highlights the first report of strains of *S. aureus* MRSA in fresh artisan cheeses capable of producing biofilms so is fundamental to determine healthy carriers engaged in the processing of the product chain and same design strategies of epidemic control or elimination of the bacterium in people who are carriers and truncating cross-contamination also implement measures of hygiene and making the Mexican rules-based product, by what is proposed to health institutions design and impart education workshops for health where emphasis on aspects as it collects milk, the hygiene measures to be used for the manufacture of the cheeses as well as for transportation, which no doubt will serve to reduce the risk of exposure to *S. aureus* by eat this type of food, this would decrease the risk of food poisoning in susceptible people who consume this product in the capital city of the State of Guerrero, Mexico.

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Conflict of interest.

The authors declare that conflict of interest for the publication of this article there is no.

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