Isolation and identification of main mastitis pathogens in Mexico

[*Isolação e identificação dos micróbios patogênicos principais
da mastite em México*]

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ABSTRACT

The present work is a large epidemiological study aiming to detect the prevalence of subclinical mastitis and to investigate the major udder pathogens in Jalisco State, western Mexico. For this purpose, 2205 dairy cows, representing 33 Mexican dairy herds, were involved. Of 2205 cows, 752 mastitic animals were diagnosed and only 2,979 milk samples could be obtained for further investigation. All 2979 milk samples were subjected to California Mastitis Test (CMT) to differentiate clinical cases from subclinical ones where 1996 samples (67 %) reacted positively. Of these, 1087 samples (54.5%) came from cows suffering from clinical cases of mastitis. Bacteriological identification of the causative agents revealed the presence of a major group of pathogens including the Coagulase negative staphylococci (CNS), *S.aureus*, *S.agalactiae*, *Corynebacterium spp*. and Coliform bacteria which were detected in 464 (15.6%), 175 (5.9%), 200 (6.8%), 417 (14%) and 123 (4.1%) of the 2927 investigated quarters, 295 (15.4%), 118 (15.7%), 111 (14.8%), 227 (30.2%) and 109 (14.5%) of the 752 examined cows and in 33 (100%), 22 (66.7%), 19 (57.6%), 30 (90.1%) and 27 (81.8%) of the 33 herds involved, respectively. Other pathogens could be detected in the investigated milk samples such as *S. dysgalactiae* (0.4%), *S.uberis* (0.37%), Bacillus spp. (1%), Nocardia spp. (0.6%) und Candida spp. (0.1%). Meanwhile, others were present in a negligible ratio; including the *Aerococcus viridans*, and Enterococcus spp., *Lactococcus lactis*, *S. bovis*.

Keywords: bovine mastitis, *Corynebact spp*., Mexico, *S.aureus, S.agalactiae*

RESUMO

O trabalho atual é um estudo epidemiológico que objetiva detectar a predominância da mastite subclínica e investigar os micróbios patogênicos principais do úbere no México ocidental. Com esta finalidade, foram utilizadas 2205 vacas leiteiras, representando 33 rebanhos de leiteiras mexicanas. Além dessas 2205 vacas, 752 animais com mastite foram diagnosticados, considerando-se que somente 2979 amostras do leite poderiam ser obtidas para a posterior investigação. Todas as 2979 amostras do leite foram submetidas ao teste da mastite de Califórnia (CMT) para diferenciar casos clínicos dos subclínicos, visto que 1996 amostras (67%) reagiram positivamente. Além dessas, 1087 amostras (54.5%) vieram das vacas que sofrem de casos clínicos de mastite. A identificação bacteriológica dos agentes causais revelou a presença dos Staphylococcus *negativos para coagulase (CNS),* S. aureus, S. agalactiae, *outros spp.* Streptococcal, Corynebacterium *spp., e as bactérias de coliformes foram detectadas em 464 (15.6%), 175 (5.9%), 200 (6.8%), 109 (3.9%), 417 (14%) e em 123 (4.1%) dos 2927 quartos investigados; em 295 (15.4%), 118 (15.7%), 111 (14.8%), 95 (12.6%), 227 (30.2%) e em 109 (14.5%) das 752 vacas examinadas e, finalmente, em 33 (100%), 22 (66.7%), 19 (57.6%), 30 (90.1%), 30 (90.1%) e em 27 (81.8%) dos 33 rebanhos envolvidos, respectivamente.*

Palavras-chave: *mastite bovina*, Corynebact *spp.*, *México*, S.aureus, S. agalactiae

INTRODUCTION

Mastitis is one of the major diseases in the veterinary field. The worldwide losses due to the disease are approximately $35 billion annually. In addition to the huge direct and indirect economic losses, the presence of certain pathogens in the milk threatens public health (Bedolla e Castañeda 2003; Bilal *et al*., 2004; Wolter *et al*. 2004).

Mastitis is a complex disease with multiple predisposing factors and various causative agents (Bergdoll 1989, Harmon 1996; Bedolla, and Castañeda 2003; Castañeda 2009; Tollersud *et al*. 2000). In Mexico, where nearly 1.9% of the global milk production takes place, the dairy industry has an important share in the domestic product (More, 2009). Therefore, mastitis control is one of the major economical challenges in Mexico. The great economic losses can be reduced only by adopting effective management and control programs. For this purpose and to ensure effective mastitis control, regular and large scale screening studies must be performed periodically to monitor the actual situation in dairy herds. In one of the very few investigations studying the causative agents of mastitis in Mexico, different bacterial species could be isolated from mastitic milk samples including *S.aureus, S.agalactiae, Mycoplasma spp*., *S. uberis*, and CNS (Castañeda *et al*. 2002; Miranda *et al*. 2008; Castañeda 2009). The prevalence of *S. aureus* in different countries varies greatly. It ranges from 3.0 - 3.5% in Finland and Germany and from 34.2% − 38.5% in Zimbabwe and Australia, (Daniel *et al.*, 1982; Myllys *et al.*, 1998; Kloppert *et al.*, 1999; Kudinha and Simango, 2002; Milne *et al.,* 2002). In other countries such as USA, Ethiopia, and Brasil the *S. aureus* was detected in 7.6%, 9.1% and 9.5%, respectively (Bishi *et al.* 1988, Wilson *et al.* 1997, Benites *et al.,* 2002). The aim of the present work is to monitor the actual role of different pathogens in the induction of mastitis in Jalisco State, west Mexico and to detect the incidence of subclinical mastitis in relation to clinical cases.

MATERIALS AND METHODS

In the present work, 2205 dairy cows (33 herds) were examined for mastitis. The majority of the cows (95% of the animals) belonged to the Holstein Friesian race while the remaining (5%) belonged to the Jersey, Brown Swiss, Criollo, and Simmental races. The California Mastitis Test was applied to differentiate subclinical cases from healthy ones. A total of 752 mastitic animals were diagnosed and consequently sampled. With the exception of 41 quarters, sufficient milk samples were obtained from the remaining 2,979 quarters. The milk samples were collected under complete aseptic conditions and the samples were kept primary at 4°C till being examined within a maximum 12h after sampling. The samples were centrifuged at 2000 x g for 30min. as recommended by Yousof – Beighi *et al.* (2005). The sediments were cultured in Blood agar. The grown colonies were subjected to different bacteriological and molecular biological identification processes. All the herds involved originate from 33 dairy herds in the State of Jalisco, Mexico. The farms represent all territories of Jalisco in order to offer a regional epidemiological map of the causative agents – genotypes of mastitis in the state.

The milk samples were cultured in sheep blood agar plates with blood agar basis (Merck, Darmstadt, Germany). The plates were prepared according to the instructions from the manufacturer, autoclaved for 15min at 120°C and left to cool to 50°C approximately. After adding 5% sterile de-fibrinated sheep blood, the liquid agar was then poured in sterile Petri dishes. Before culturing, the samples were warmed to room temperature, mixed thoroughly and centrifuged at 2000 x g for 30min. The sediment was taken for this purpose. Finally, the inoculated plates were incubated under aerobe conditions at 37°C and read after 24 h and 48h, respectively.

In addition to colony morphology and type of hemolysis produced, suspected colonies were also identified by Catalase Test and Tube Coagulase Test (Harmon *et al.* 1990; Brückler and Schwarz, 1994). The results obtained through the biochemical identification were confirmed by the application of species specific PCR. The API Staph Strip- and the API 20 Strep systems (API System S. A., Montalieu-Vercieu, France) were used to identify the isolated colonies. The systems were used according to the recommendations from the manufacturer.

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The polymerase Chain reaction (PCR) was applied for the molecular confirmation of the results obtained through the biochemical identification. For this purpose, the *S. aureus* species specific primer pair Staur4 (5'- ACG GAG TTA CAA AGG ACG AC -3') and Staur6 (5'-AGC TCA GCC TTA ACG AGT AC-3'; MWG Biotech; Ebersberg, Germany) was used for the partial amplification of the 23S rRNA gene with an amplicon size of 1270 base pairs (bp) according to Straub *et al.*, 1999. The suspected colonies were suspended, subcultured in blood agar and incubated overnight at 37°C. For the extraction of bacterial DNA, 5-10 colonies were suspended in 100μL TE buffer before adding 5μL lysostaphin (1.8U/μL) (Sigma, Deisenhofen, Germany). Subsequently, the mixtures were incubated at 37°C for 1h to enhance cell wall lysis. Followed by the addition of 10μL proteinase K (14.8mg/mL, Roche, Mannheim, Germany), the tubes were then re-incubated at 56°C for 2h. The samples were then boiled for 10min in order to inactivate the proteinase K. Finally, the mixtures were centrifuged (1min, 10,000g) and the supernatant was frozen at -20°C till being used as a template for the PCR reactions according to El-Sayed *et al.* (2005).

The reaction mixture (20μL) contained 12.9μL bidest, 0.7μL of both forward and backward primers (10pmol/μL), 0.4μL of dNTP (Biozym, Germany), 2μL of 10X PCR buffer (Sigma, Germany), 1.2μL of MgCl2 (25mmol, Sigma), 0.1μL of Taq DNA polymerase (5U/μL, Sigma). Finally, the template DNA (2μL) was added separately in each reaction tube. The reaction types were then put into the thermocycler (T3 Thermocycler, Biometra, Germany). The PCR products were visualized in a 2% agarose gel (Roth, Karlsruhe, Germany) in 35 mL TBE buffer (40mmol/l Tris, 1% [v/v] acetic acid, 1 mmol/l EDTA). The gels were loaded with 8μL of the PCR product mixed with 3μL of sample buffer (50% glycerine, 50 mmol/L EDTA, 0.25% bromphenole-blue). In addition to the samples, a marker was added to the gel (GeneRulerTM DNA Ladder Mix, MBI Fermentas, St. Leon-Rot; 100Bp DNA Ladder, Gibco/BRL, Eggenstein; DNA length standard VIII, Roche, Mannheim; DNA length standard VI, Roche). The gels were subjected to electrophoretic separation for 1h at 100 V before being stained with Ethidium Bromide for 20min (5μg/mL

Sigma, Deisenhofen, Germany). The gels were evaluated under UV light (gel documentation system Image Master, VDS, Pharmacia Biotech, Freiburg, Germany).

RESULTS

In the present work, milk samples from healthy, subclinical and clinically mastitic cows in 33 herds of dairy cattle (n= 2205 cows) in Jalisco, Mexico were investigated. According to the clinical examination and the CMT results in the studied herds, 752 mastitic animals were diagnosed. Of these mastitic animals, 41 quarters were sever inflamed so they yielded insufficient amounts of milk at the sampling time, therefore were excluded from the statistics. The other 2979 sampled quarters could be divided into healthy quarters (n=983), subclinically mastitic quarters (n=1942), clinically mastitic quarters with mild to moderate inflammation (n=54), and clinically mastitic quarters with severe inflammation (n=41). The results are summarized in Table 1. All 2979 milk samples were tested with the California Mastitis Test (CMT). Of these, 1996 samples (67%) reacted positively to CMT. While 1087 (54.5%) of the 2979 samples originated from clinically mastitic cows, only 21 samples (0.7%) showed milk clots and 33 (1.1%) showed changes in color. The majority of the samples 2925 (98.2%) were apparently normal. While no bacterial growth could be detected in 53% of the samples, the grown colonies from the rest of the samples were identified using different tests. These included the analysis of the type of haemolysis in blood agar, results of Api Staph and APi-20 Strept System and Catalase and Coagulase Tests.

For the differentiation between *S. aureus* and the other Coagulase negative staphylococci, beside the results of the Coagulase test, the staphylococcal colonies were subjected to the partial amplification of the *S. aureus* specific gene 23S rRNA. The identification of the cultures revealed the presence of different milk pathogens in the samples. Namely; Coagulase negative staphylococci (CNS), *S.aureus, S.agalactiae*, *Corynebacterium spp*. and Coliform bacteria were detected in 464 (15,6%), 175 (5,9%), 200 (6,8%), 417 (14%) and 123 (4,1%) of the 2927 investigated quarters, 295 (15,4%), 118 (15,7%), 111 (14,8%), 227 (30,2%) and 109 (14,5%) of the 752 examined

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cows and finally in 33 (100%), 22 (66,7%), 19 (57,6%), 30 (90,1%) and 27 (81,8%) of the 33 involved herds, respectively. In addition to these, the *S. dysgalactiae* (0,4%), *S.uberis* (0,37%), Bacillus spp. (1,0%), Nocardia spp. (0,6%) und Candida spp. (0,1%) could be also detected. Other uncommon pathogens as *Aerococcus viridans*, *Enterococcus spp.*, *Lactococcus lactis*, *S. bovis* could also be isolated.

All the *S. aureus* strains isolated coud be identified by PCR with the amplification of the specific gene 23S ARNr (Figure 1).
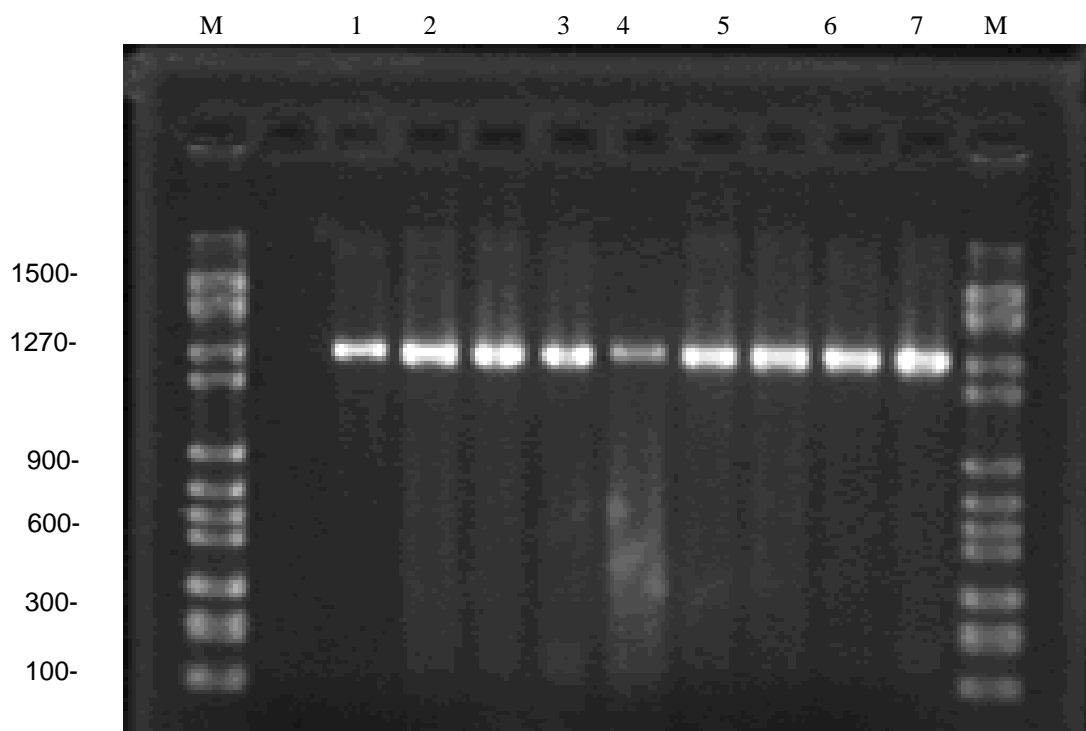

Figure 1. Enlargement of the species specific area of the 23S rRNA gene of *S. aureus* with a uniform enlargement of 1,270 base pairs. M = molecular weight marker.

Table 1. Subdivisions of milk samples according to physical properties, bacteriological examination and CMT

Milk yield	milk samples (n=2979)	No milk (n=41)
Normal milk (physically)	Normal (n=2925)	Abnormal (n=54)
Normal milk (bacteriologically)	no growth (n=1603)	Growth (n=1376)
Normal milk (CMT)	no clumps (n=983)	Clumps (n=1996)

DISCUSSION

Although screening of the actual mastitis situation in dairy herds is a very important step in disease control, a very limit number of monitoring studies in Mexico could be found in literature. In the present work, a large monitoring investigation was performed in order to detect the incidence of unremarked subclinical mastitic cases and to detect the main pathogens affecting udder health in Jalisco State, West Mexico. To improve the detection level of the bacteria present in the milk samples, centrifugation of the sampled milk was done at first as recommended previously (Yousof – Beighi *et al.,* 2005). Bacteriological identification of the major causative agents mainly detected the presence of Coagulase Negative Stahpylococci*,* followed by *Corynebact. spp.*, *S.agalactiae*, *S.aureus*, Coliform bacteria, and other bacteria which seem to play a minor role as mastitis inducers such as *S. dysgalactiae*, *S.uberis*, *Bacillus spp*., *Nocardia* spp. *Candida spp.* and *Aerococcus viridans*, Enterococcus spp., *Lactococcus lactis, S. bovis.*

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The detection level of these bacteria differed according to the type of milk sample, herd and region. This difference in the epidemiological role of different pathogens in the induction of mastitis according to the type of mastitis, herd conditions and the region was also reported by other teams (Castañeda *et al*, 2002; Medina 2002; Sommerhäuser, 2002; Da Silva *et al.,* 2005; El-Sayed 2006; Eissa 2009). A recent study conducted in central Mexico reported the presence of *S. aureus* in 30%; *S. uberis* in 37.5% and CNS in 38.3% of the investigated dairy cows (Miranda *et al.,* 2008). The clear variation in the epidemiology of mastitis and mastitis inducers among different regions in Mexico is surprising. In the present data *S.aureus* was detected in 15.7% of investigated cows (30% in central Mexico) while the *S.uberis* seems to play a none significant role in mastitis (37.5% in central Mexico). Yet these differences may be attributed to other factors rather than geographical location, such as the differences in the samples taken or type of mastitis.

The dominance of *S. aureus* as the most prevalent contagious mastitis pathogen is also seen in USA and Mexico, where it was isolated from 43% of bulk milk samples followed by *S. agalactiae* which was isolated from 3% of the samples. The high prevalence *S. agalactiae* indicates poor management in investigated farms according to Kloppert *et al*. (1999). In opposition to our data, *S. agalactiae* was rarely isolated (0.8% and 0.12%) from mastitic milk according to Busato *et al*., (1997) and Myllys *et al*. (1998), respectively. *Mycoplasma* was isolated more frequently in larger herds (Lombard *et al.,* 2008). In the investigated region, the phenotypic β-haemolysing field strains seem to be of decisive importance as the field strains show a statistically higher significant virulence potential.

CONCLUSION

The present work offers epidemiological data regarding the situation of mastitis among dairy herds in Jalisco State. The prevalence of both subclinical and clinical mastitis and the role of different mastitis inducing pathogens were discussed.

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