

## **DETECTION OF THE *mecA* GENE IN STRAINS OF STAPHYLOCOCCUS AUREUS METHICILLIN RESISTANT ISOLATED FROM MILK SAMPLES OF DAIRY COWS THROUGH THE POLIMERASE CHAIN REACTION (PCR)**

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### **Abstract**

In the present study, milk samples of 25 dairy cows were taken from a herd known to be positive to *Staphylococcus aureus* (*S. aureus*). On parallel experiments milk samples were screened for the presence of *S. aureus* through standard bacteriological procedures and for the presence of the *nuc* gene (*S. aureus* specific) through PCR performed straight from DNA isolated from the milk samples. All *Staphylococcus* strains isolated were also tested for the presence of the *nuc* gene and challenged with penicillin, ampicillin and oxacillin in an *in-vitro* sensitivity test through the Kirby-Bauer protocol. Those strains resistant to the latter antibiotic were used in an oxacillin minimum inhibitory concentration (MIC) test, where each strain was cultured independently in Muller-Hinton agar enriched with 4% NaCl with increasing concentrations of oxacillin ranging from 0.5 to 240 mg/ml. The milk samples positive to methicillin resistance *S. aureus* (MRSA) strains were used to determine the presence of the *mecA* gene, which has been associated to bacterial multi-resistance to antibiotics. *S. aureus* were isolated through bacteriological protocols and the gene *nuc* was detected through PCR from 18 (72%) out of the 25 milk samples. *Staphylococcus epidermidis* was isolated from three other milk samples which were negative to the *nuc* gene through PCR. Four milk samples were negative to either species of *Staphylococcus* on bacteriological culture and to the *nuc* gene through PCR. Nineteen (90.4%) out of the 21 strains isolated were resistant to penicillin and ampicillin and 7 (33.3%) were resistant to oxacillin. From those bacteria resistant to oxacillin 2 (28.5%) were positive to the *mecA* gene through PCR. The same milk samples from where the *mecA* positive strains were isolated, were positive to the *nuc* and *mecA* genes through PCR.

### **INTRODUCTION**

Pathogenicity and virulence of the *Staphylococcus aureus* (*S. aureus*) can vary considerably among strains and since it is a normal inhabitant of skin and mucous membranes in man and animals, the outspread of dangerous strains is not difficult (Barkema et al., 1998, Tenover et al., 1994; Zadocks et al., 2000;). In the last two decades the increasing number of *S. aureus* strains resistant to several antibiotics has become a public health problem mainly in hospitals (Kloos and Bennerman, 1995). One of the factors of the *S. aureus* reported as responsible for antibiotic multi-resistance is the *mecA* gene, which is responsible for the synthesis of the penicillinase binding proteins (PBPs) (Reynolds, 1988). *S. aureus* strains carriers of this gene become resistant to methicillin, a third generation antibiotic resistant to betalactamases, and has been associated to resistance to other antibiotics (Seguin et al., 1999). This gene plays an important role of the natural

race between antibiotic development by the pharmaceutical industry and bacterial resistance development to antibiotics. In order to win this race to the bacteria, it is imperative to develop strategies that allow identification of potentially dangerous strains of bacteria. *S. aureus* has been reported world wide as one of the main opportunistic infectious agents affecting mammary gland in dairy cows (Gonzalez et al., 1988). It has also been reported the presence of methicillin resistant *S. aureus* (MRSA) strains in the milk dairy herds (Devriese and Hommez, 1975). Since this strains of *S. aureus* could outspread easily through direct contact with milk or milk products that have not been pasteurized and through material or people that is in touch with the contaminated milk and or any other contaminated material in the dairy farm, it is of outmost importance to know the status that dairy herds have with respect to and the to the MRSA carriers of the *mecA* gene.

Bacteriological isolation and characterization of MRSA strains is not enough to determine if the strain is a carrier of the *mecA* gene since there have been some reports of some MRSA that are negative to the *mecA* gene. It is possible to determine through a very highly sensitive test based on PCR, if a particular MRSA strain is a carrier of the *mecA* gene (Vannuffel et al., 1995). Although it is a good alternative to isolate characterize MRSA strains and then determine through PCR if they are carriers of the *mecA* gene. However It could become a very laborious and intensive job to characterize all the strains that could be present in a single cow through this method. Hence, herein we describe a diagnostic protocol based on PCR to determine if a particular cow is positive to a *mecA* MRSA strain.

## MATERIALS AND METHODS

As control for all genetic and phenotypic analysis three reference strains were used: *S. aureus* ATCC25923 (methicillin sensitive, *mecA* negative), *S. epidermidis* ATCC12228 (methicillin sensitive, *mecA* negative) and a field strain of *S. aureus* N° 305 characterized by our laboratory (methicillin resistant, *mecA* positive).

Milk samples from twenty five dairy cows were taken aseptically directly from the nipples previously washed, disinfected with ethanol (70%) and dried with individual sterile paper towels. Milk was taken from each nipple (2.5ml) and pooled in a sterile recipient. Milking was performed manually and a fresh pair of latex gloves was used for each animal. Five microliters of milk from each cow were seeded on an blood agar and mannitol-salt agar plates and cultured overnight to detect the presence of *S. aureus*. One colony of each mannitol-salt plate was characterized through bacteriological standard techniques (gram staining, colony morphology, carbohydrate fermentation and coagulase test). Each colony was tested for the presence of the *nuc* gene through PCR (as described later), tested for *in-vitro* sensitivity to penicillin (10IU/ml), ampicillin (15 µg/ml), cephalotin (30 µg/ml) and oxacillin (methicillin analog) (1.0 µg/ml) through the Kirby-Bauer protocol according to the NCCLS (1997). Those Colonies resistant to oxacillin in the Kirby-Bauer sensitivity test were tested through a minimal inhibitory concentration test (MIC) and analyzed for the presence of the *mecA* gene through PCR as described later. MIC test was performed as described by Thornsberry and Mc Dougal (1983) and the NCCLS (1997). Briefly: Staphylococcus strains resistant to oxacillin were seeded in a fresh plate of mannitol-salt agar and grown overnight, five colonies were pooled and seeded on one ml of Muller-Hinton broth supplemented with 4% NaCl and cultured for four hours, 10 µl from each strain were sedded separately on a plate of Muller-Hinton agar supplemented with 4% NaCL at with different concentrations of oxacillin: 0.5, 1.5, 3, 6, 12, 24, 120 and 240 µg/ml. Each strain was tested per triplicate).

DNA purification from milk was performed according with the protocol reported by Khan et al. (1998), briefly: Each sample of milk was treated independently. One ml of milk was transferred

to a 1.5 ml microcentrifuge tube and centrifuged at 13,600 x g for one minute, supernatant was discarded and the pellet was resuspended with PBS and centrifuged again at 13,600 x g for one minute. This step was repeated five times. Pellet was resuspended and vortexed vigorously in 22  $\mu$ l of lysis buffer (tris-EDTA NaCl and 3  $\mu$ l of a solution 1M of NaOH), warmed at 95°C for 15 minutes and kept at -20°C until use.

PCR analysis: *nuc* gene was amplified using the primers SA1 (5' gcgattgatggtgatacggtt 3') SA2 (5' agccaagccttgacgaactaaagc 3') and the thermocycling parameters (94°C for four min one cycle; 94°C one min, 55°C for 30 sec and 72°C for 1.5 min, 37 cycles; 72°C for 3.5 min one cycle) described by Khan et al. (1998). Diagnostic band for the *nuc* gene is of 270 bp.

*mecA* gene was amplified using the primers mec1 (5' tggctatcgtgtcacaatcg 3') mec2 (ctggaacttgttgagcagag 3') and the thermocycling parameters (92°C for three min one cycle; 92°C one min, 56°C for one min and 72°C for one min, 30 cycles; 72°C for 2 min one cycle) described by Vannuffel et al. (1995). Diagnostic band for the *mecA* gene is of 310 bp. PCR reactions were loaded in a 2% agarose gel with ethidium bromide and run for 30 min at 70 volts, visualized on a UV transilluminator and analyzed by a Kodak DC120 system.

## RESULTS AND DISCUSSION

The reference strains used as control showed to be phenotypically and genotypically as expected.

From the 25 milk samples 21 (84%) were contaminated with *Staphylococcus* spp. Eighteen (72%) of the colonies were characterized phenotypically as *S. aureus* and three (28%) as *S. epidermidis*. From the 21 strains, 20 (95.2%), 19 (90.4%), 2 (9.5%) and 7 (33.3%) were resistant to penicillin, ampicillin, cephalotin and oxacillin respectively. From the seven colonies resistant to oxacillin and tested in the MIC test 4 (57.14%) demonstrated sensitivity at a concentration of 120  $\mu$ g/ml, and the other 3 (42.86%) at 1.5  $\mu$ g/ml of oxacillin. From the 21 strains isolated, the same 18 colonies classified phenotypically as *S. aureus* were positive to the *nuc* gene by PCR, and the other three were negative. These results demonstrate that the species characterization of the strains found in this study was correct. It is interesting to point out that the bacteriological diagnostic was performed in 48 hours while the PCR diagnostic was made in 8 hours. The pattern of antibiotic resistance is shocking since mostly used antibiotics in the field to treat mastitis are penicillin and ampicillin and most strains isolated are resistant to both of them. It is also interesting to observe that some strains show some resistance to cephalotin and oxacillin so those antibiotics should be used carefully.

From the 7 strains resistant to at least 1  $\mu$ g/ml of oxacillin six were *S. aureus* and one *S. epidermidis* and from the four strains resistant to at least 24  $\mu$ g/ml of oxacillin three were *S. aureus* and the other one *S. epidermidis*. To our knowledge this is the first report of a *S. epidermidis* resistant to oxacillin isolated from dairy milk.

From these same 7 strains resistant to 1  $\mu$ g/ml of oxacillin two were carriers of the *mecA* gene, both strains were *S. aureus*. It is noteworthy that this two strains were also resistant to all other antibiotics used in this experiment, which confirm that the risk of widespread of the *mecA* positive *S. aureus* strains from the dairy herd to human and other animals is present. From the result found in the present work we confirmed that not all the MRSA strains are carriers of the *mecA* gene.

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