# Genetic Characterization of Resistant S. Aureus Strains Isolated From Cow's Milk with Mastitis

**Healthcare**

**Keywords:** MRSA, cattle, antibiogram, mecA.


| | | |
|---|---|---|
| **Keti Margariti** | | **Veterinarian, Tirana, Albania** |
| **Pranvera Cabeli** | | **Faculty of Veterinary Medicine, Agricultural University of Tirana, Albania** |
| **Domenico Buonavoglia** | | **Universita degli Studi Aldo Moro, Facolta Medicina Veterinaria, Bari, Italy** |
| **Jonida Boci** | | **Institute of Food Safety and Veterinary, Tirana, Albania** |
| **Sonila Cocoli** | | **Faculty of Veterinary Medicine, Agricultural University of Tirana, Albania** |
| **Tana Shtylla** | | **Faculty of Veterinary Medicine, Agricultural University of Tirana, Albania** |
| **Aida Dama** | | **Albanian University, Tirana, Albania** |



## Abstract

Mastitis therapy is generally unsuccessful due to pathological changes that occur in the breast as a result of the inflammatory response, factors associated with the causes of mastitis, the pharmacokinetic properties of antimicrobial drugs, etc. The main reason for the low effectiveness of antibiotic treatment of mastitis caused by staphylococci is especially, among other things, the resistance of bacteria, where is recognized that Staphylococcus aureus is the first penicillin-resistant bacteria. Laboratory techniques based on phenotypic characteristics of resistance have low sensitivity. PCR (Polymerase Chain Reaction) as a method for detecting gene mecA is considered as gold standard test. Detection of mecA gene through PCR is one of the most effective methods for the detection of MRSA. Using this method we identified six methicillin-resistant strains of S. aureus isolated from cow's milk with mastitis. Our study presents the first survey carried out in our country regarding the presence of the mecA gene for antibiotic-resistant strains of S. aureus which is isolated from cow's mastitis. This study has a special importance for food products of animal origin, like milk contaminated with MRSA may constitute a main source of consumer's infection.


## 1. Introduction

The treatment of cow's mastitis is related to the nature of the etiological agent, susceptibility of antibiotics, clinical signs, immune system and biological condition of the body. Bacteriological diagnosis is not sufficient to have an effective treatment of mastitis, whether bacterial agents isolated from milk samples are not tested for its sensitivity to antimicrobial preparations. Numerous studies conducted in this area have concluded that microbial agents isolated from clinical cow's mastitis will not differ in sensitivity to antibiotics from microbial agents isolated from sub-clinical cases of mastitis. Among the in-vitro sensitivity tests, antibiogram (agar diffusion technique) represents the most common of the tests when we seek to identify the most effective antibiotics against recognized pathogens in order to install a successful therapy.

Mastitis therapy is generally unsuccessful due to pathological changes that occur in the breast as a result of the inflammatory response, factors associated with the causes of mastitis, the pharmacokinetic properties of antimicrobial drugs, etc. The main reason for the low effectiveness of antibiotic treatment of mastitis caused by staphylococci is especially, among other things, the resistance of bacteria, where is recognized that Staphylococcus aureus is the first penicillin-resistant bacteria. Laboratory techniques based on phenotypic characteristics of resistance have low sensitivity. PCR (Polymerase Chain Reaction) as a method for detecting gene mecA is considered as gold standard test (Éva Juhász-Kaszanyitzky 2007).

The nature of resistance to methicillin and the presence of mecA gene are less studied in animals, especially when it comes to strains isolated in the herds. Detection of mecA gene through PCR is one of the most effective methods for the detection of MRSA. In this paper, the in-vitro sensitivity to methicillin, for all strains of staphylococci isolated in pure culture from cases of mastitis in dairy cows, was studied by the genotypic method (PCR) for detection of mecA gene, as responsible for resistance to β- lactam antibiotics.

**2. Material and Methods**

The antimicrobial susceptibility to S. aureus isolated from clinical and sub-clinical cases of mastitis was tested by agar diffusion method.S. aureus strains isolated from sub-clinical and clinical cases of mastitis in cows, in the study areas, were tested for antimicrobial susceptibility to panel of the following antimicrobials: augmentine, cephalexin, cefuroxime, ceftasidine, kotrimosazol, gentamycin, tetracycline and ac. Nalidixic.

Techniques used (antibiogram) refer to well-known model of Kirby-Bauer, which is based on the deposition of a number of cellulose disc, previously saturated with a determined quantities of antibacterial preparations, which are placed in a Petri's dish with the solid terrain, where the previously pathogen's strains are planted.

Antibiotic sensitivity determination was based on the international standard diagram, which estimates the size of areas for each level of sensitivity and their interpretations for each antibiotic as follows:

Susceptibility (S): the microbial agents should react to treatment with antibiotics to the normal doses

Intermediate sensitivity (I): the microbial agents should not respond to treatment at normal level, but should react to higher doses than normal if this dose can be given with certainty.

Resistant (R): the microbial agents is impossible to respond to the treatment with specific antibiotics.

For the evaluation of the results achieved, depending on the parameters of the inhibition's zone of growth, we have carried out with four crosses system, according to which crosses 0-1, microbial strain was considered R (Resistant), 2-3 SI (Intermediate Susceptibility) and 4 crosses, microbial culture was named S (Susceptibility).

Also, in this paper are presented the results on the presence of MRSA strains isolated from bovine mastitis on farms in the study. Were tested a total of 24 strains of staphylococci isolated in pure culture from cases of mastitis in cows. Milk's samples taken from cows with mastitis in aseptic manner was used for isolation of S. aureus strains. After 48 hours in terrains planted noted the growth gram positive and catalase positive cocci. Identified as staphylococci strains were tested for the presence of coagulase and biochemical profile of the identified strains was determined by means of API 20 Staph system. Biomerieux, France. Coagulase test was carried out using rabbit plasma, according to the instructions of the diagnostic source (Biokar Diagnostics, Beauvais, France). Also, all of the S. aureus strains isolated from cows with mastitis were planted in Mannitol Salt Agar (MSA) (OXOID, Milan) for 48 hours at 37°C in aerobic conditions.

PCR for gene MECA was carried out according to the methodology described by Murakami et al, 1991, using the set of primers:mecA1:

5'–AAAATCGATGGTAA-AGGTTGGGGC-3' mecA2: 5' GCAAATCCGGTACTGCAGAAC-3',

That is amplified in gene fragment 533bp mecA. PCR was carried out in a final volume of 50 µl using DNA Thermal Cycler Gene Amp 9600 (Perkin Elmer Cetus, Norwalk, CT, USA). 5 DNA microliter mixture joined the reaction as follows: PCR buffer (10X) 1.5 mM MgCl2;200 mM of each oligonucleotide trifosfatase; 0.1 mM

of each primer; 2.5 UI of Amplitaq Gold polymerase (Perkin Elmer Cetus Norwalk, USA); Distilled H2O to achieve a final volume of 50 microliter.

The reaction mixture was placed in one cycle prior denatured 94°C for 8 minutes, which was followed by 35 cycles of amplification, each one from 1', is thus 1' annealing at 55°C and ended with the last cycle, and the survey was conducted in 72°C for 10 minutes. PCR products were placed in electrophoresis with 2% agarose gel and ethidium bromide.

DNA was extracted according to the technique known for Gram positive bacteria [9].

Strains were planted in Nutrient Broth (NB, Liofilchem) and were placed in the thermostat overnight in aerobics conditions at 37°C. 1 ml of the culture liquid was centrifuged and pellet were obtained by rinsing it out 2 times. After centrifugation at 1,000 laps in 10', the extract obtained was used as template for PCR test

**3. Results and Discusion**

The results of sensitivity or resistance of staphylococci identified from acute or sub-clinical cases of mastitis, to antibiotics cited below, are presented in the following table:

Table 1. Susceptibility of Staphylococcus aureus to antibiotics

| Antibiotics | Staphylococcus aureus | | | |
|---|---|---|---|---|
| | n | s % | I % | R % |
| Chloram. | 9 | 55 | 3 | 42 |
| Norfloxacin | 22 | 59 | 14 | 27 |
| Enrofloxacin | 20 | 60 | 25 | 15 |
| Tetracycline | 24 | 42 | 21 | 27 |
| Oxytetracycline | 17 | 35 | 41 | 24 |
| Neomycin | 13 | 64 | 21 | 15 |
| Flumequine | 17 | 41 | 24 | 35 |
| Erithromycin | 20 | 46 | 21.2 | 32.8 |
| Trimeth. Sulf | 6 | 33 | 29 | 38 |
| Amoxicillin | 16 | 18 | 32 | 50 |
| Amikacin | 5 | 20 | 17 | 63 |
| Cefalexin | 20 | 40 | 15 | 60 |
| Penicillin | 20 | 25 | 10 | 65 |
| Kanamycin | 15 | 66 | 20 | 14 |
| Oxacillin | 8 | 30 | 22 | 48 |
| Ampicillin | 11 | 36 | 9 | 55 |
| Streptomycin | 10 | 30 | 20 | 50 |
| Gentamcin | 17 | 53 | 31 | 16 |
| Lincomycin | 6 | 17 | 16 | 67 |

We note that the efficiency of medical treatment of bovine mastitis depends on the agent's causative, clinical signs, and susceptibility to antibiotics of etiologic agents. Furthermore, treatment of mastitis with antibiotics usually has poor results, due to the pathological changes that occur in the breast as a result of the inflammatory response, factors associated with causative bacteria, the pharmacokinetic properties of antimicrobial drugs, etc.

Therefore, as seen in the above results, the main reason for the low effectiveness of antibiotic treatment of mastitis caused by staphylococci, is among other things, the resistance of bacteria, which is in this case, is considered multiple. When a bacterial strain emerged resistant to more than three classes of antibiotics, it is characterized by a multiple resistance. Moreover, microorganism which has developed resistance to many antibiotics commonly used for treating mastitis, now recognized as the first bacterium resistant to penicillin is Staphylococcus aureus. It has been observed that strains of Staphylococcus aureus isolated from mammalian secretions of inflamed gland are more resistant to antibiotics commonly used to treat mastitis than coagulase-negative strains of staphylococcus.

In-vitro susceptibility testing to antibiotics is considered as a predictor of treatment results of intramammary infections caused by Staphylococcus aureus and other pathogens. This is because the efficiency of antibacterial treatment to breast pathogens depends not only in their introduction in the gland tissue through intramammary route, but it is also associated with various indigenous factors present in milk, such as immunoglobulins, lacto peroxidase, etc (Dwight C Hirshet al 1999).

Consequently many studies show that the most significant factor that affects the speed of treatment of clinical mastitis caused by Staph. aureus, is a capacity of Staph. aureus strains to produce the enzyme β-lactamase, which are activated by the inflammatory process born in mammalian gland.

Interaction between antibiotics and immune factors may lead to increased antibacterial effect in infected tissues, or otherwise reduce it. Certainly, the elimination of mastitogens requires the interaction of two factors, the effectiveness of antimicrobial preparations and good functioning of the immune system of the animal. The sustainability of infections caused by staphylococci is certainly favored by the easiness with which these bacteria gain antibiotic-resistance. Resistance can be stimulated by chromosomal mutations or through the transfer of genome fragments (Livermore D M 2000). PCR techniques performed on the S. aureus strains isolated from mastitis cows highlighted the presence of gene mecA. 18 strains planted in terrain MSA gave the distinctive yellow color in this terrain, which expresses a biochemical feature of their ability to ferment mannitol associated this with S. aureus. This test were negative for 6 strains of staphylococcus isolated from animals with mastitis. MSA negative strains were tested with the test of coagulase and resulted coagulase-negative.

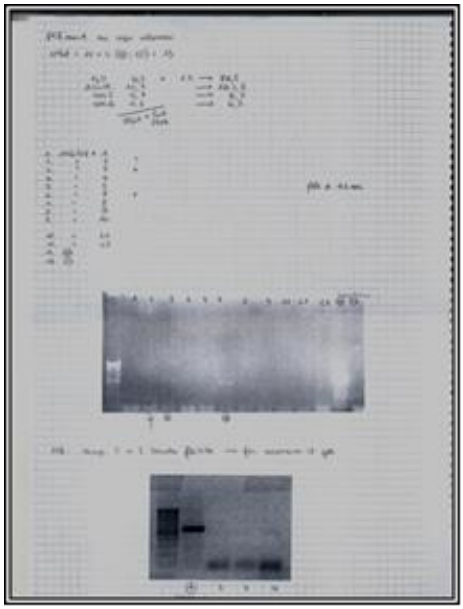
Staphylococci cultures increase in MSA (yellow color culture shows strains of S. aureus)

Six strains of staphylococcus, which resulted coagulase negative, were tested with the API system, for the determination of Staphylococcus species. These strains of staphylococci, isolated from mastitis in cows were resulted to: Staph. hyicus encoded 6506051(2 strains) and Staph. chromogenes encoded 67160 7/5 2 (4 strains).

Figure 1. Staphylococci cultures increase in MSA (yellow color culture shows strains of S. aureus)

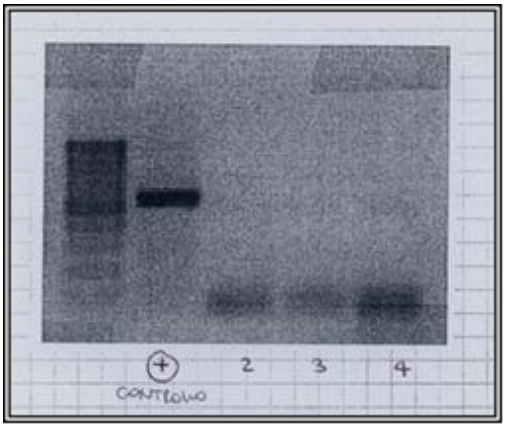
All strains of S. aureus were tested for the presence of gene mecA by PCR technique. In the following figure are given protocol analysis of the work performed for the strains in the study.

**Figure 2.** Working protocol and results of PCR for gene mecA



Strains analyzed through PCR technique showed that six of them were positive for mecA gene, after amplification occurred at 533bp fragment mecA gene, and after S. aureus control strain ATCC showed his product 533bp.

**Figure 3.** Results for the presence of mecA gene




The results of our study confirms the high percentage of MRSA strains isolated from mastitis of cows. The techniques used in this study has allowed us to characterize the nature of the strains of staphylococci methicillin-resistant. In particular, the presence of mecA gene identified through PCR technique is an indicator of the presence of β-lactamases strains in milk of cows with clinical or sub-clinical mastitis (Fluit A C et al 2001; Marina Morgan 2008). Our results also confirm that for detection of mecA gene, PCR is a "gold" test to detect true methicillin resistant strains. We can conclude that 25% of strains of staphylococci isolated in this study were strains of "true" methicillin-resistant, in accordance with accepted classical definitions (Chambers HF et al 1997; Tauxe R.V 2002). This result is consistent with other studies where mecA strains were isolated from cases of mastitis in cows. Many researchers think that perhaps mecA strains isolated from animals with mastitis are

associated with those with human nature (Tauxe R.V 2002; York M K et al 1996). Production of β-laktamases is an important indicator for determining the antibiotic-resistance. For this reason, it is very important to conduct a process of monitoring of antibiotics resistance in the case of mastitis in cows, through various in-vitro tests, (Marina Morgan 2008; Massidda O et al 1996). Our results indicate that specific PCR for detection of gene mecA is the appropriate method for identification of "true" resistance to methicillin in S. aureus isolates of animal origin. This study represents the first signal for the presence of the MRSA in cow's milk with mastitis in our country. This study has a special importance for food products of animal origin, like milk contaminated with MRSA may constitute a main source of consumer's infection (Éva Juhász-Kaszanyitzky et al 2007). Our study presents the first survey carried out in our country regarding the presence of the mecA gene for antibiotic-resistant strains of S. aureus which is isolated from cow's mastitis.

## 4. Conclusions

Staphylococcus aureus strains isolated from mastitis of cows has presented resistance in Linkomycin, Penicillin, Amikacin, Ampicillin, Streptomycin, Amoxillin, etc.

Molecular typing of MRSA is an important step of control programs of infection caused by S. aureus. The presence of mecA gene identified through PCR technique is an indicator of the presence of strains of β-lactamases S. aureus in milk of cows with clinical or sub-clinical mastitis.

25% of strains of staphylococci isolated strains resulted "true" meticillin-resistant. Our results confirm that PCR is a "gold" test to detect true strains methicillin-resistant of S. aureus.

This study represents the first signal for the presence of MRSA in milk of cows with mastitis in our country and recognition of the current prevalence of MRSA constitutes a duty for future preparation and research.

## 5. References

1. Chambers HF: Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clinical Microbiology 1997, 10: 781-791.
2. Dwight C Hirsh and Yan Chung Zee: Veterinary Microbiology 1999
3. Éva Juhász-Kaszanyitzky, Szilárd Jánosi et al: MRSA Transmission between Cows and Humans, Emerging Infectious Diseases 2007, 13: 45-56
4. Fluit A C, Visser M R, Schmitz F J: Molecular detection of antimicrobial resistance. Clinical Micobiology Reviews 2001,14: 836-871.
5. Kitai S, Shimiziu A, Kawano J, Sato E, Nakano C, Kitagawa H: Characterization of methicillin-resistant Staphylococcus aureus isolated from retail raw chicken meat in Japan. J Vet Med Sci 2005, 67:107- 110.
6. Livermore D M: Antibiotic resistance in staphylococci. Int. J.Antimicrobic agents 2000, 16:S3-S10
7. Marina Morgan: Methicillin-resistant Staphylococcus aureus and animals: zoonosis or humanosis? Journal of Antimicrobial Chemotherapy 2008, 62: 1181–1187
8. Massidda O, Montanari M P, Mingola M, Varaldo P E: Borderline methicillin-suspectibile Staphylococcus aureus strains have more in common than reduced susceptibility to penicillase-resistant penicillins. Antimicrobic Agents Chemotherapy 1996, 40: 2769-2774.
9. Tauxe R.V: Emerging foodborne pathogens. International Journal of Food Microbiology 2002, 78: 31-41
10. York M K, Gibbs L, Chehab F, Brooks G F: Comparison of PCR dection of mec A with standart susceptibility testing methods to determinate methicillin resistance in coagulase-negative staphylococci. Journal of Clinical Microbiology 1996, 34: 249-253.