Prevalence and Antimicrobial Susceptibility Profiles of Pathogen Isolated from Bovine Mastitis Milk in Transylvania, Romania

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Abstract

Mastitis in cows, one of the most common and economically important infectious diseases of dairy cattle, all over the world, with significant impact due to economic losses, occurs when the udder becomes inflamed because the leukocytes are released into the mammary gland usually in response to bacteria invasion of the teat canal.

The main objective of this study was to evaluate the *in vitro* antimicrobial susceptibility of bacteria isolated from milk in order to design specific control programs for bovine mastitis in this area.

A total of 204 milk samples aseptically collected both from farms and private owners were processed during May 2014 and March 2016 within the Microbiology Laboratory of the Faculty of Veterinary Cluj-Napoca, Romania. The microbiological examination was carried out by inoculation on blood agar and MacConkey medium. After the overnight incubation in aerobic conditions, the identification of the isolates was performed using microscopic, cultural and biochemical methods. Biochemical identification was based on API 20 Biomerieux system. Susceptibility to antibiotics was evaluated using Kirby Bauer disk diffusion method on Mueller-Hinton agar; the antibiotics were represented by Amoxicillin and Clavulanic Acid, Ceftiofur, Florfenicol, Mastidiscs, Enrofloxacin, Penicillin and Tetracycline.

Staphylococcus spp. was the most common isolated pathogen, in 54.9% of the specimens, followed by *Streptococcus* spp. in 20.1%, *Escherichia coli* in 10.78%, *Klebsiella* spp. in 8.34%, *Bacillus* spp. in 5.88%. The most frequent associations were represented by staphylococci-streptococci in 62.7% of the samples, followed by streptococci-bacillus in 19.8% of the samples. The most important etiological agents identified were *Staphylococcus aureus*, *S uberis*, *Streptococcus agalactiae*, and *Escherichia coli*. Antimicrobial susceptibility test for the total isolates revealed good sensitivity to Enrofloxacin, Mastidiscs and Amoxicillin and Clavulanic Acid. Resistance was observed for Penicillin and Tetracycline.

The major mastitis pathogens identified was *Staphylococcus aureus*, while recurrent mastitis treatment was based on systemic and local administration of Enrofloxacin and Mastidiscs respectively.

Keywords: *antimicrobial agents, bovine mastitis, etiology, milk*

INTRODUCTION

Bovine mastitis is one of the most important, complex and costly pathology of the dairy industry with substantial impact on the economy of milk production. Due to its high incidence the economic impact is related to treatment cost, losses production and changes in the milk quality. The complexity is reflected in the variety of gram-positive and gram negative etiological agents (Bogni *et al.,* 2011; Cervincova *et al,* 2013). Mastitis is produced by a wide spectrum of pathogenic microorganisms that penetrate the teat canal and

multiply in the udder cistern and it can be clinical and subclinical (Carrillo, 2012, Sztachańska *et al.*, 2016).

Clinical mastitis caused by Gram-positiv bacteria such as: *Staphylococcus aureus Streptococcus agalactiae* and *Streptococcus dysgalactiae*, *Streptococcus uberis* and Gram–negative pathogens such as: *Escherichia coli*, *Klebsiella* spp., or *Pseudomonas* spp., and occurred more often in herds with a high bulk milk somatic cell count (SCC) (Barkema *et al.*, 1998). Other pathogens such as: *Pseudomonas* spp., *Bacillus* spp., *Pasteurella* spp., yeasts (mainly *Candida albicans*) and algae are occuer infrequently.

Three type of clinical mastitis exists: peracute, acute and sub-acute and it is charactherised by the presence of gross inflammation signs, while in the sub-clinical form no signs of gross inflammation or milk abnormalities can be observed. Changes in milk composition can be detected by special diagnostic tests.

Mastitis is one of the most important cow pathology and the treatment and prevention of this diseases is a frequent reason that antibiotics are administered to cows (Oliveira *et al.*, 2014; Saini *et al.*, 2012).

The present study was conducted to identify the etiology of clinical mastitis and to evaluate the *in vitro* antimicrobial susceptibility of some Gram-positive and Gram-negative bacteria isolated from milk in order to developed mastitis control strategies, in this area.

MATERIALS AND METHODS

The research has been carried out during may 2014-march 2016, within the Microbiology Laboratory, Faculty of Veterinary Medicine Cluj-Napoca, Romania. A total of 204 mastitic milk samples collected in separated sterile samples collected tubes, both from farms and private owners were examined. The microbiological investigation, for the colony characteristic was carried out by inoculation on blood agar and MacConkey agar plates. After 24 hour incubation in aerobic conditions, the identification of the isolates was performed using microscopic, cultural and biochemical methods.

The microscopic examination of the bacteria was done using Gram staining method (Shina, 2006; Subha, 2016). Biochemical identification was based on API 20 Biomerieux system (Bio

Mérieux, France). The antibiotic sensitivity was performed by Kirby Bauer difusimetric method on Mueller Hinton agar plates, using bioMérieux disc diffusion; the antibiotics tested were represented by Amoxicillin and Clavulanic Acid, Ceftiofur, Florfenicol, Mastidiscs, Enrofloxacin, Penicillin and Tetracycline.

RESULTS AND DISCUSSION

Phenotipic identification of etiological agents were performed follow bacteriological examination of the milk.

The colonies developed on culture media were innitialy morphologically evaluated, than from each colonie type, microscopic preparation stained by Gram method were performed (Tab. 1).

The genera and the bacterial species identification were made by the API Staph, API 20 Strep and API 20 E systems (Fig. 2). The most frequent bacterial genus who belong the isolated etiological agents were presented in the Fig. 1.

One of the major mastitis pathogen *Staphillococcus aureus* was isolated from 112 (54.9%) of samples (Fig. 2). Among streprococci, so calles mastitis streptococci, *S. uberis* and *S. agalactiae* predominated, occurring in 41 (20.1%) of samples. The most prevalent streptococcus mastitis was *S. uberis* identified in 95% of cases, foloweed by *S. agalactiae* (5%). Also infection of the mammary gland caused by *E. coli* and *B. cereus* play an important role. Regarding coliform enterobacteria, *E. coli* prevailed in 22 samples (10.78%) and *Klebsiella* spp. was identified in 17 samples (8.34%). Other pathogen such as *Bacillus cereus* occurring in 12 (5.88%) of samples.
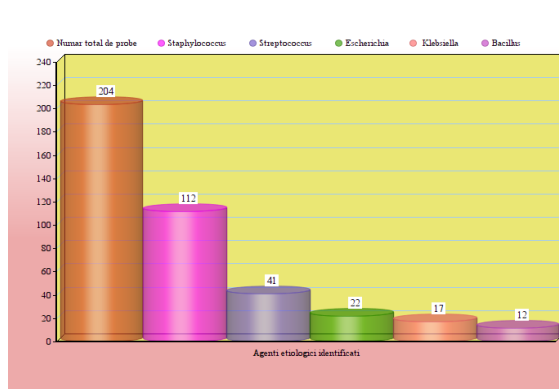
The results of our study concerning the incidence of etiologic agents in clinical bovine mastitis are in accordance with the existing literature data (Cervinkova *et al.*, 2013, Fiţ *et al.*, 2010, Matei, 2010). Also in Poland similar results were reported by Malinowski *et al.* (2006) and Bochniarz *et al.* (2013).

Staphilococcus aureus is the major etiological agent of bovine mastitis. In our study for the total number of the examined samples, this pathogen was isolated from 112 (54.9%) of milk samples. This is in accordance with the other reported data (Cervinkova *et al.*, 2013, Sztachańska *et al.*, 2016).

Antimicrobial susceptibility. Table 2 summarise the medium of diameter inhibition area (mm) of 7 antibiotics tested for each identified etilogic agent.

Tab. 1. The results obtained to bacterioscopic identification of bacterial species

Total no. of the examined samples	The colonies appearance	Bacterioscopic exam	Bacterial genus
112	1-3 mm diameter, smoth, glossy and golden pigmented colonies	G+, round (cocci), grape-like cluster	*Staphylococcus*
41	pointy colonies	G+, cocci in chains	*Streptococcus*
22	medium in size, transluced colonies, pink coloured on MacConkey agar	G- rods, with no particular cell arrangement	*Escherichia*
17	glossy, mucoid, 3-4 mm diameter colonies	G-, rod-shaped, singly and in pairs or short chains	*Klebsiella*
12	3-4 mm diameter colonies, irregular edges, flat, opaque, adherent to the culture medium	large G+ bacilli, diplo and chains	*Bacillus*
204			

Fig. 1. The frequency of the bacterial genus who belong
the isolated etiological agents.

Fig. 2. API Staph system Biomerieux: *Staphylococcus aureus* biochemical identification.

In general, *S. aureus* isolates were susceptible to the majority of tested antibiotics (Table 2). Resistance to Penicilline G and Tetracycline was most frequently observed for *E. coli* and *Klebsiella* spp isolates whereas resistance to other antibiotics was sporadic and observed only with Amoxicillin and Clavulanic acid for *Klebsiella* spp. isolates and Ceftiofur for *Bacillus cereus* isolates (Table 2).

Antibiotics have been used routinely for the treatment of cows mammary gland pathology and also for preventing other new infections. But, while the antibiotic therapy has an important role reducing the incidence of mastitis, the emergency of antibiotic-resistant pathogens has produced serious damages.

Our data obtained to the antimicrobials susceptibility test (Table 2) showed that the most efficient antibiotics were:
- Tetracycline for *S. aureus* isolates;
- Amoxicillin and Clavulanic Acid for *S. uberis* isolates;
- Enrofloxacin for *E. coli* and *Klebsiella spp.* isolates;
- Florfenicol for *B. cereus*.

Regarding the resistance, the less efficient antibiotics were represented by:
- Ceftiofur for *S. aureus* isolates;
- Mastidiscs for *S. uberis* isolates;
- Penicillin G and Tetracycline for *E. coli* isolates;
- Penicillin G,Tetracycline and Amoxicillin and Clavulanic Acid for *Klebsiella spp.* isolates;

Tab. 2. The medium of diameter inhibition area (mm) of 7 antibiotics tested for each identified etilogic agent (*n=202*)

Antibiotics tested	The antibiotics concentration (µg/disc)	The etiologic agent				
		S aureus (n=112)	*S.uberis (n=39)*	*E.coli (n=22)*	*Klebsiella spp. (n=17)*	*Bacillus cereus (n=12)*
		Medium of inhibition area (mm)				
Amoxicillin and Clavulanic acid	30	20	23	20.5	R	18.5
Ceftiofur	30	19	21.3	17	11	R
Florfenicol	30	21.7	22.5	20.3	19	19.3
Mastidiscs	30	20.3	17.6	21	16	18
Enrofloxacin	5	20.11	19.8	22	20	19.2
Penicillin G	10	19.3	20.5	R	R	13.6
Tetracycline	30	23.3	20	R	R	17.5

R = resistant isolate, n=number of tested strains

• Ceftiofur for *B. cereus* isolates.

One of the important problem in the treatment of bovine mastitis is the antimicrobial resistance. In the last period, an increasing of the antimicrobial resistance rate has been registered in *S. aureus* from bovine mastitis (Saini *et al.* 2012; Wang *et al.* 2013). On the one hand the resistance is a result of inappropriate antibiotic and chemotherapeutics administration via the intramammary route both by individual breeders as well as intensively, and on the other hand preventive adiminration of drugs, leading to establishing the antibiotic resistance phenomenon.

Although in the last time significant progress were performed in the treatment and control of mastitis in cattle, research for developed the new therapeutic alternatives is one of the current challenges in the field, however, the incidence of these infection continue to remain highly. In this context further investigation in the field required, for obtaining a quality milk, according to European Union standards, with any risk to consumer health.

CONCLUSION

1. The results of our study revealed that the incidence of clinical mastitis in this area is high and the most frequently isolated pathogens were: *Staphylococcus aureus* (54,9%), *S. uberis* and *S. agalactiae* (20.1%), *E. coli* (10.78%), *Klebsiella* spp. (8.34%) and *Bacillus cereus* (5.88%).

2. The major bovine mastitis pathogens was *St2. aphylococcus aureus*, while recurrent mastitis treatment was based on systemic and local administration of Enrofloxacin and Mastidiscs respectively.

3. Antimicrobial susceptibility test for the total isolates revealed good sensitivity to Enrofloxacin, Mastidiscs and Amoxicillin and Clavulanic Acid.

4. The less efficient antibiotics were Penicilline G and Tetracycline. This is due to improper establishment and administration of antibiotic both in therapy and as preventive measure, leading to the antibiotic resistance.

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