Antimicrobial Susceptibility Profile of Bacteria in Mastitis Cow Milk Samples

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RESEARCH ARTICLE

Abstract

Bovine mastitis is a cause of major financial losses in the dairy industry worldwide. Bovine mastitis is defined as the inflammatory response of the udder tissue due to either physical trauma or infections caused by different microorganisms, especially bacteria. More than 135 pathogenic agents have been identified from bovine mastitis. The aim of the study was to identify the bacterial genera involved in mastitis etiology in cattle and to test the antimicrobial susceptibility to antibiotics. The samples were collected before morning milking and evaluated by microscopic and cultural examination, and further identified using biochemical methods. The evaluation of antimicrobial susceptibility was performed using Kirby-Bauer disk diffusion test. Overall, *Staphylococcus* spp., *Bacillus* spp., *Streptococcus* spp., *Micrococcus* spp., *Escherichia* spp. and *Trueperella* spp. were identified. The most frequent bacterial associations were observed for *Staphylococcus-Bacillus*, *Bacillus-Streptococcus* and *Staphylococcus-Micrococcus*. Regarding the antimicrobial sensitivity profile, the majority of the bacterial strains we have isolated were classified as sensitive, with the exception of *Escherichia coli*, with one strain being resistant to all tested antibiotics. Among the antimicrobials, the Amoxicillin and clavulanic acid was the most efficient one and Trimethoprim/Sulfamethoxazole did not exhibit an inhibitory activity in 19 milk samples.

Keywords: cow milk; bovine mastitis; AMR, antimicrobial susceptibility.

INTRODUCTION

Mastitis is a cause of major financial losses in the dairy industry worldwide. These losses are mainly due to reduced yield and poor milk quality caused by changes in milk composition (Botrel et al., 2010, Cheng and Han, 2020). The significant economic impact translates into decreased milk production, discarded milk and pre-term drying off. Moreover, cow mastitis is also associated with premature culling and even death (Gomes and Henriquez, 2016). Bovine mastitis is defined as the inflammatory response of the udder tissue due to either physical trauma or infections caused by different microorganisms, especially bacteria (Cheng and Han, 2020). More than 135 pathogenic agents have been identified from bovine mastitis (Abdi et al., 2021). Bacteria that are commonly involved in the etiology of cow mastitis are classified as either contagious or environmental pathogens. Contagious pathogens are transmitted from cow to cow most often during milking time, whereas the second category of bacteria have their reservoir in the animals' environment, in the bedding and housing of the herd (Royster and Wagner, 2015). Major contagious mastitis pathogens are represented by *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma* spp. On the other hand, the environmental pathogens group includes a wide range of microorganisms, such as coliforms (*E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp.), *Trueperella pyogenes*, non-aureus staphylococci (NAS), streptococci (*Streptococcus dysgalactiae*, *Streptococcus uberis*) and other bacteria such as *Pseudomonas* spp., *Proteus* spp., *Aerococcus* spp., yeasts or algae (*Prototheca* spp.) (Abdi et al., 2021).

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Unlike contagious pathogens, environmental ones do not usually live on the cow's udder and teat skin, thus they are considered opportunistic microorganisms (Cameron et al., 2016).

Based on the degree of inflammation, bovine mastitis can be classified into 3 major groups: clinical, subclinical and chronic mastitis. Clinical mastitis evolves with visible signs and abnormal milk secretion, whereas subclinical mastitis shows no visible changes in the udder or milk (Barlow et al., 2011). In this case, milk production is often reduced and the milk somatic cell count is significantly increased. Conversely, chronic mastitis is defined as a long term inflammatory process that can last for several months. Nevertheless, the subclinical form accounts for more financial losses compared to clinical mastitis, due to the fact that it is quite difficult to quantify because it evolves with no visible signs and the diagnosis requires further milk testing (Cheng and Han, 2020). The most frequently isolated pathogens from clinical cases of mastitis are *Escherichia coli*, *Streptococcus uberis* and *Staphylococcus aureus*, whereas subclinical mastitis is most often caused by coagulase-positive staphylococci (CPS), NAS and streptococci. *E. coli* is less frequently involved in the etiology of subclinical forms (Persson Waller et al., 2011). However, these four major groups (coliforms, CPS, NAS, streptococci) represent a real concern for the public health, as they can play an important role as reservoir for antimicrobial resistance genes and, moreover, they can be involved in spreading the genes to other pathogenic and commensal microorganisms in the farm (Boireau et al., 2018).

Because dairy cattle mastitis is mainly caused by bacteria, antibiotics are an important component of the therapeutic approach and control programs (Boireau et al., 2018). Thus, intramammary infections are the most common cause for antimicrobial use in lactating cows. Due to this excessive use of antibiotics, resistant bacterial strains have been isolated from bovine mastitis in the last few years (Alekish et al., 2013). Surveillance of the antimicrobial resistance is extremely important in order to minimize the risk for selection of resistant bacterial strains and also limit the spread of antimicrobial resistance which poses a serious threat for the public health system, making this problem a global concern (Persson Waller et al., 2011). In recent years, there has been observed a growing interest in the emergence and diffusion of antibiotic resistance that results from the aggressive use of antimicrobials in the veterinary sector.

The phenomenon of antimicrobial resistance associated with bovine mastitis pathogens has two relevant aspects. The first one is related to a low cure rate after the treatment of clinical cases and the second one is the potential transmission of resistant microorganisms to humans via the food chain (Sol et al., 2000; Pinzón-Sánchez and Ruegg, 2011). Nevertheless, this transmission is possible whenever subclinical mastitis is involved, due to the presence of resistant strains in bulk tank milk that may be passed to humans via raw milk (Tenhagen et al., 2006). The literature is abundant in studies focused on antimicrobial resistant bacteria isolated from bovine mastitis (De Oliveira et al., 2000; Tenhagen et al., 2006; Pitkala et al., 2008; Oliver and Murinda, 2012; Liu et al., 2014; Thomas et al., 2015).

In this context, the aim of this paper is to identify the bacterial genera involved in mastitis etiology in cattle and to test the antimicrobial susceptibility to antibiotics.

MATERIALS AND METHODS

A total number of twenty-one milk samples from cattle diagnosed with clinical mastitis collected from three farms in Alba County have been included in the study. All samples were collected before morning milking, after teats end cleaning with 70% ethanol. First streams of foremilk were discarded and milk was collected aseptically in sterile plastic containers. Specimens were processed the same day, refrigerated during transportation and had modified consistency, aspect and colour. Milk samples have been initially evaluated by microscopic and cultural examination, and further identified using biochemical methods. Microscopic examination involved stained smears using Gram staining technique, and offered details regarding the shape, size and arrangement, being considered useful for orienting the next step, cultural examination.

The culture media used were represented by blood and Sabouraud agar. In this concern, a loopful of milk was used to inoculate blood agar medium and Sabouraud agar. Blood agar plates were incubated at 37°C for 18-24h, while Sabouraud agar plates at 30°C for 48-72h. The cultural examination is conferring details regarding the size, shape and colour of the colonies. The type of hemolysis (alpha, beta, gamma) was determined on blood agar plates and the identification of bacteria was performed by evaluating biochemical characters (catalase, oxidase and hyaluronidase). Therefore, a catalase test was conducted on Gram-positive cocci to differentiate staphylococci from streptococci and the oxidase test was utilized to differentiate the *Enterobacteriaceae* from Gram-negative non-*Enterobacteriaceae* bacteria. Oxidase negative microorganisms were further inoculated on MacConkey agar for the evaluation of lactose fermentation. Milk samples with two different colonies were considered a mixed infection.

The evaluation of antimicrobial susceptibility was performed using Kirby-Bauer disk diffusion test. In this concern, Mueller-Hinton agar plates have been flooded with 1 ml from the suspension of mixed flora isolated colonies in broth, at the density of 0.5 on McFarland scale, using the electronic density evaluator Densicheck™ Plus (Biomérieux). The antibiotics were represented by amoxicillin and clavulanic acid (AMC) 20/10µg, cefotaxime (CTX) 30µg, doxycycline (DO) 5µg, enrofloxacin (ENR) 5µg, florfenicol (FFC) 30µg, gentamicin (GEN) 10µg, penicillin (P) 10UI and trimethoprim/sulfamethoxazole (STX) 25µg. Breakpoints for zone diameter in mm were considered as follows: AMC 22, CTX 20, DO 22, ENR 18, FFC 17, GEN 21, P 26 and STX 22.

RESULTS AND DISCUSSIONS

Bacteria were identified in twenty (95.23%) out of twenty-one milk samples evaluated. From a total of nine milk samples a single bacterial genus was identified, whereas in the remaining eleven milk samples, bacterial associations were present. Individual bacteria identified in mik samples were represented by *Staphylococcus* spp. (n = 4), *Bacillus* spp. (n = 2), *E. coli* (n = 2) and *Trueperella* spp. (n = 1). Overall, *Staphylococcus* spp. was identified in a total of nine samples, *Bacillus* spp. in 8, *Streptococcus* spp. in 5, *Micrococcus* spp. in 3, while *E. coli* and *Trueperella* spp. each in 2 samples. All samples were negative for yeast growth on Sabouraud agar. The results regarding the bacterial associations are presented in Table 1.

Table 1. The bacterial associations identified in milk samples

Bacterial associations	Number of milk samples
<i>Staphylococcus</i> , <i>Bacillus</i>	3
<i>Bacillus</i> , <i>Streptococcus</i>	3
<i>Staphylococcus</i> , <i>Micrococcus</i>	2
<i>Staphylococcus</i> , <i>Trueperella</i>	1
<i>Staphylococcus</i> , <i>Streptococcus</i>	1
<i>Streptococcus</i> , <i>Micrococcus</i>	1

For the antimicrobial susceptibility testing, the overall results revealed that for the majority of milk samples included in the study, at least one antibiotic was efficient and recommended. The most efficient antimicrobial was represented by AMC, as only seven bacterial isolates from five samples were resistant. AMC was also recommended for the treatment of eight cattle, the best in this concern. P and ENR also demonstrated good overall results, each of these antibiotics being recommended to be used in order to treat four cows. GEN was recommended in the treatment of two cattle, while DO for one.

Table 2. The number of resistant bacterial strains for each tested antibiotic

	<i>Staphylococcus</i> spp.	<i>Streptococcus</i> spp.	<i>Bacillus</i> spp.	<i>Micrococcus</i> spp.	<i>Trueperella</i> spp.	<i>E. coli</i>	Total
AMC	2	1	2	1	0	1	7
CTX	1	2	1	2	0	2	8
DOX	2	1	2	0	0	2	7
ENR	1	1	1	1	0	2	6
FFC	1	0	1	1	0	1	4
GEN	1	2	1	1	0	1	6
P	4	2	3	1	0	2	12
STX	8	5	7	3	2	2	27
Total	20	13	18	10	2	13	

From the total of twenty milk samples evaluated for antimicrobial susceptibility testing, six bacterial isolates were resistant to GEN, seven to AMC and FFC, seven to ENR, eight to CTX, ten to DO and 19 to STX. Regarding multidrug resistance (MDR), the strains were so considered if the resistance was recorded for at least three antimicrobial substances from three different classes (Magiorakos *et al.*, 2012). The only MDR strain was *E. coli*, with total resistance for all tested antibiotics for one strain, whereas the second isolated strains showed resistance to five out of eight antimicrobials. Thus, CTX, DOX, ENR, P and STX had no efficiency against this strain of *E. coli*. Furthermore, from all antibiotics included in the study, STX showed moderate inhibitory activity in case of one strain from the total of twenty evaluated and no activity for all the other. The results regarding the resistance to antimicrobials for each genus are presented in Table 2.

Therefore, from a total of twenty positive milk samples, we have isolated 6 bacterial genera, as follows: *Staphylococcus* spp., *Bacillus* spp., *Streptococcus* spp., *Micrococcus* spp., *Escherichia* spp. and *Trueperella* spp. The results of our study are consistent with other papers in the literature focused on bovine mastitis (Tenhagen *et al.*, 2006, Persson *et al.*, 2011). Although the prevalence of udder pathogens may vary between countries, studies generally describe staphylococci, streptococci and *E. coli* as the major pathogenic agents involved in the etiology of clinical mastitis (Botrel *et al.*, 2010; Thomas *et al.*, 2015). However, in recent years, farmers have started to implement different measures in order to prevent intramammary infections, such as milk hygiene, better farm management and an appropriate use of antimicrobials. Such measures tend to reduce the incidence of mastitis. Moreover, a change in the hierarchy of mastitis pathogens can be observed. For example, *Staphylococcus aureus* and *Streptococcus agalactiae* seem to have a lower prevalence nowadays, but on the other hand, CNS and *Trueperella* spp. are more frequently isolated from mastitis milk samples (Zhang *et al.*, 2018). However, in our study the most encountered bacterial genus was *Staphylococcus* spp., followed by *Bacillus* spp.

and *Streptococcus* spp., showing that both staphylococci and streptococci are still major causative agents of clinical bovine mastitis in Romania, our results being in accordance with other studies which obtained a similar prevalence for *Staphylococcus* spp., *Streptococcus* spp. and *E. coli* (Tenhagen et al., 2006; Persson et al., 2011). Regarding other Gram-positive bacteria, a study conducted by Tenhagen et al. (2006) focused on the prevalence of mastitis pathogens in dairy cows from Germany, revealed a higher prevalence of *Trueperella* spp. compared to the results of the present research. On the other hand, the same authors isolated *Enterococcus faecalis* and *Enterococcus faecium*, but with a much lower frequency, 0.2% compared to other strains (Tenhagen et al., 2006). Abdi et al. (2021), in a study conducted on both clinical and subclinical mastitis in the USA, showed that the most frequently isolated pathogens from cases of clinical mastitis were *E. coli*, *Klebsiella oxytoca* and *Streptococcus dysgalactiae*, with a significantly higher percentage of *E. coli* strains (79.4% of all *E. coli* were isolated from clinical mastitis) compared to the results of our study. Conversely, the same study revealed that the main microorganisms involved in the etiology of subclinical intramammary infections were represented by *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Streptococcus uberis* (Abdi et al., 2021). However, in contrast with the results obtained in the above mentioned studies, the present paper also revealed the presence of *Bacillus* spp., with a significantly high prevalence regarding the milk samples included in the study (8 out of 20 positive samples).

Nowadays, antimicrobial therapy is commonly used for both mastitis prevention and control, but unfortunately, treatments failures are encountered quite often, due to the emergence of antimicrobial resistant bacterial strains (Saini et al., 2012). This resistance of mastitis pathogens to commonly used antibiotics is a well-documented problem in the industry of dairy cows and it is now being closely monitored (Tenhagen et al., 2006). Moreover, identifying the bacterial strains involved in the etiology of bovine mastitis, as well as their sensitivity to antimicrobials at the level of region or country is extremely important in order to preserve and adapt the therapeutic protocols (Botrel et al., 2010). Nevertheless, another serious threat which is closely related to the aggressive use of antibiotic in the treatment of cattle mastitis is the potential risk of transmission of resistant pathogens to humans via raw milk and raw milk products, leading to a possible public health hazard (Waller et al., 2011).

Overall, the prevalence of antimicrobial resistance was low. The most resistant bacterium isolated from the milk samples we have analyzed was represented by *E. coli*. Therefore, out of the two isolated strains, one was resistant to all tested antibiotics. The only efficient antimicrobials against this microorganism were AMC, FFC and GEN, with an inhibition zone diameter of 22.49 mm, 22.87 mm and 22.26 mm respectively. The results of our study are consistent with other studies in the literature regarding coliform mastitis and the antibiotic resistance patterns (Thomas et al., 2015, Abdi et al., 2021). The antimicrobial resistance of *E. coli* has been frequently reported in recent years, especially regarding bacterial strains isolated from cases of mastitis in dairy ruminants (Liu et al., 2014; Zhang et al., 2018). The high levels of antimicrobial resistance for this strain in particular could be explained by long-term use of these antibiotics in dairy herds, without a prior bacteriological culture of milk samples and a sensitivity test. Throughout the world, *E. coli* is one of the most important pathogens associated with cow clinical mastitis and even though it is considered an environmental agent, it is responsible for causing serious tissue damage. Moreover, the antibiotic resistance developed by this microorganism is one of the major problems whenever recommending a treatment, due to low cure rates (Liu et al., 2014).

Regarding Gram-positive cocci sensitivity results, the present study revealed a low prevalence of antibiotic resistant strains. However, the isolated strains belonging to *Staphylococcus* and *Streptococcus* genus showed a rather high resistance to P and STX, the results being in accordance with other papers (Abdi et al., 2021). The same observation is available for *Bacillus* spp. On the other hand, the most susceptible bacteria to antimicrobials was *Trueperella* spp., the strains being resistant only to STX. Overall, AMC, a frequently used antimicrobial agent, showed a good activity against both Gram-positive and Gram-negative results. However, in order to avoid the spread of antibiotic resistant bacterial strains, it is highly recommended to perform microbiological tests on milk samples, to identify the pathogens and perform the antibiotic sensitivity testing.

CONCLUSIONS

Overall, from a total of 21 milk samples collected from cows diagnosed with clinical mastitis, 20 samples were positive for bacterial growth. Thus, bacteria belonging to 6 different genera were isolated. *Staphylococcus* spp. was the most frequently isolated genus, followed by *Bacillus* spp. and *Streptococcus* spp. Other Gram-positive bacteria were represented by *Micrococcus* spp. and *Trueperella* spp., which have been identified in a small number of samples. Among the Gram-negative microorganisms, we have isolated *E. coli*, but with a low frequency. Regarding the antimicrobial sensitivity profile, the majority of the bacterial strains we have isolated were classified as sensitive, with the exception of *E. coli*, with one strain being resistant to all tested antibiotics. Among the antimicrobials, the AMC was the most efficient one and STX did not exhibit an inhibitory activity in 19 out of 20 milk samples.

Author Contributions: G.C.N. Conceived and designed the analysis; C.S.N. Collected the data; C.M.B. Contributed data or analysis tools; I.A.M. Performed the analysis; N.I.F. Provided the resources; G.C.N., C.S.N. Wrote the paper.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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