Scientific Works. Series C. Veterinary Medicine. Vol. LXVIII (2), 2022
ISSN 2065-1295; ISSN 2343-9394 (CD-ROM); ISSN 2067-3663 (Online); ISSN-L 2065-1295

ASSESSMENT OF THE ANTIBIOTIC RESISTANCE PROFILE IN MASTITIC MILK OF DAIRY COWS, DEPENDENT ON THERAPY AND CLINICAL CONDITION

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Abstract

Mastitis remains a major challenge for the global dairy industry, despite the widespread implementation of control strategies. Escherichia coli and Streptococcus uberis are the most common agents of mastitis in cattle and a significant problem for the dairy industry. The aim of this study was to establish the prevalence of bacteria involved in mastitis of dairy cows and to establish the antibiotic resistance profile, which frequently complicates the therapy. In order to achieve these objectives, samples (n=25) were collected from cattle diagnosed with clinical mastitis and subclinical mastitis detected by biochemical assays. The samples were processed by standard microbiological methods and the results indicated an increased prevalence of Staphylococcus spp. and of microorganisms of the Enterobacteriaceae family. Isolated bacterial strains have shown significant resistance to antibiotics, especially to amoxicillin, while ciprofloxacin has proven the most effective. Thus, the early detection and correct treatment of clinical and subclinical mastitis is an important challenge for the economy and for the public health.

Key words*: mastitis, E. coli, antimicrobial resistance, dairy cows.*

INTRODUCTION

Mastitis is one of the most common disease of dairy cattle associated with significant economic losses. With the advent of improved breeds and intensive breeding systems, the incidence of subclinical and clinical mastitis has increased. Cows' mastitis, as inflammatory processes of the mammary gland, are produced by different causative agents, in most cases they are microbial agents, that can be classified into three groups: specific microorganisms that cause exclusively mastitis (*Sreptococcus agalactiae*, etc.); microorganisms that affect the whole body and can also be located in the mammary gland (*Mycobacterium bovis, Mycobacterium tuberculosis, Brucella abortus, Echerichia coli*, *Pasteurella multocida*, *Micoplasma* spp., etc.); and occasional pathogens that can cause accidental mastitis (*Corynebacterium pyogenes, Pseudomonas aeruginosa Staphylococcus epidermidis*, *Staphylococcus aureus, Actinomyces* spp., *Arcanobacterium pyogenes, Proteus* sp., *Prototheca* spp., *various yeasts,* etc.) (Dworecka-Kaszak et al., 2012; Cheng and Hang 2020). The incidence of the isolation of different bacterial species involved in the etiology of mastitis is varied and depending on several factors, among which the conditions of exploitation, the applied therapy and the physiological period of the mammary gland are of particular importance (Quinn et al., 2015; Lakew et al., 2019). Numerous observations have shown that the lactating mammary gland is more susceptible to *Streptococcus agalactiae* and *Staphylococcus aureus*, while the in dry period the mamary gland is more susceptible to *Streptococcus dysgalactiae, Streptococcus uberis, Actinobacillus pyogenes* infections (Belay et al., 2022, Lakew et al., 2019). The main component of pathogenicity *S. agalactiae* is the virulence, however, it can be also pathogenic through the secretion of hemolysin, streptokinase, hyaluronidase, protease and deoxyribonuclease. Bacterial proliferation is rapid and occurs in the epithelium of the galactophorous ducts, rarely being accompanied by penetration into the canalicular and acinar epithelium. As a result, neutrophils

are transferred from the blood capillaries to the galactophorous ducts, sometimes associated with a neutrophilic reaction (Burvenich et al. 2007).

The inflammatory process blocks the lumen of the galactophorous ducts and causes alveolar involution, replacement of secretory tissue with fibrous connective tissue, atrophy, hypogalaxy and agalaxia. Mastitis are typically chronic or subclinical, with occasional acute outbreaks and the pathogenic process being limited to the mammary gland. *Streptococcus dysgalactiae* has been isolated from the tonsils, rumen and genitals of cows as well as from the skin of the mamary gland or from their mammary secretions. It usually produces subclinical forms of mastitis. *Streptococcus uberis* usually produces subclinical mastitis and persists for long periods of time (Radsak et al., 2000).

The aim of this paper was to evaluate the interrelationship of subclinical mastitis and the microbiome status for diagnosis, prognosis and therapy. In this regard, milk samples were collected and analyzed from cattle diagnosed with mastitis to establish the bacterial prevalence involved in such pathological processes. In addition to the isolation and identification of bacterial species involved in the appearance of mastitis, the antibiotic resistance profile was also evaluated in order to achieve an appropriate prognosis and therapeutic protocols.

The results of these tests can be used to monitor, prevent and treat correctly and early pathological processes in the mammary gland.

MATERIALS AND METHODS

The milk samples (n=15) from private Fleckvieh herd were collected after screening quarters using the R-Mastitest. Milk samples from healthy cows (n=5) were also collected. For initial microbiological analyses, the samples were inoculated in nutrient broth, Chapman and MacConkey agar (both from Oxoid) in aerobic conditions at 37°C for 24 hours. Bacterial strains identification were performed by standard microbiological methods adopted from the Clinical and Laboratory Standards Institute (CLSI) guideline. The identification of microorganisms was performed using Api Staph 20 and Api 20E identification galleries.

The antimicrobial sensitivity patterns of the isolated strains were evaluated using the standard Kirby-Bauer disk diffusion method according to the CLSI guidelines. The strains were tested towards 8 antimicrobials: amoxicillin/clavulanic acid (AMC, 20/10 µg) amoxicillin (AX, 10 µg), streptomycin (S 10 µg), cefoperazone (CFP, 30 µg), ciprofloxacin (CIP, 5 µg), oxytetracycline (OT 30 µg), penicillin (P, 10UI), neomycin (N, 30 µg); all purchased from Oxoid. The results were assessed based on the growth inhibition zone diameters (mm), and were calculated also the multiple antibiotic resistance index according to Krumperman (Krumperman 1983). According to the standard values of CLSI the tested strains were classified as sensitive (S), Intermediate resistant (I) and resistant (R).

RESULTS AND DISCUSSIONS

Of a total of 15 (n = 15) milk samples collected from cows diagnostised with subclinical mastitis 16 different single bacterial colonies were obtained. The isolated *strains* were identified as *Staphylococcus xylosus* (15.38%), *Staphylococcus lentus* (23.07%), *Shigella* spp. (7.69%), *Actinobacter bauman* (7.69%), *Cryseomonas luteola* (7.69%), *Enterobacter cloacae* (30.76%), *Echericia vulneris* (7.69%) (Fig. 1).

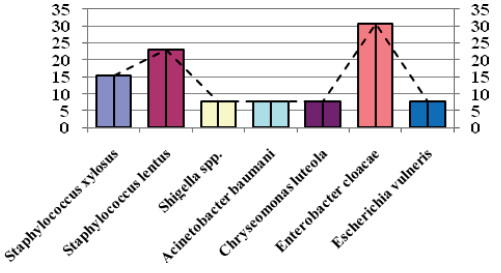

Figure 1. Percentage distribution of strains isolated from cow's milk with mastitis

From healthy cows *Staphylococcus xylosus* (30%), *Staphylococcus sciuri* (60%) and *Staphylococcus lentus* (10%) were isolated (Fig. 2).

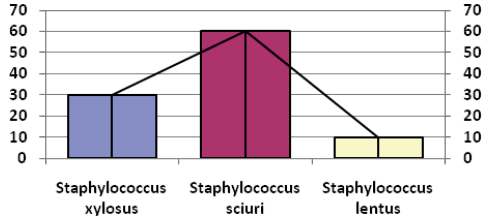

Figure 2. *Staphylococcus strains* isolated from healthy cow's milk (%)

Subclinical mastitis caused by intra-mammary infection with coagulase-negative staphylococci is one of the most common causes of dairy cows. The control of this infection is often complicated due to the increased number of bacterial species that are isolated in these cases. A study of 11 flocks, testing milk samples from affected quarters at one-month intervals and identifying bacteria isolated by biochemical tests, showed that persistent intra-mammary infection were characteristic of quarters infected with *Staphylococcus epidermidis, Staphylococcus chromogenes*, and *Staphylococcus simulans*. No differences were established between coagulase-negative staphylococcal species in relation to daily milk production, somatic cell count and month of lactation in cows with subclinical mastitis. *S. epidermidis* has been identified mainly in pluriparous cows with intra-mammary infection, while *S. chromogenes* has been isolated mainly from primiparous cows with intra-mammary infection (Thorberg et al., 2009). The isolated strains were evaluated for their level of antibiotic resistance, the results are indicated in Table 1. Of the total isolated strains, 93.75% showed total resistance to Amoxicillin/Clavulanic acid, 25% to Penicillin 31.25% to Neomycin and Streptomycin, 12.5% to Amoxicillin and Oxytetracycline, and 68.75% to Cefoperazone. The MAR index can be used to facilitate the interpretation of the results. This represents the ratio between the antibiotics to which the tested strain is resistant and the total antibiotics used for the antibiogram. The closer the MAR value is to 1, the more resistant the strain is to the antibiotics used for evaluation (Fig. 3).

The prevalence of more than 60% of bacteria with a MAR index greater than 0.4 is closely related to the therapeutic history of the study animals. Antimicrobials are often used in the treatment of the mastitis in large ruminants, so the possibility of antibiotic resistance should be considered. According to the results of our study in all isolated strains the MAR index was 1. Our results are not in line with the studies performed by Oliveira and coworkers (2012) who indicate a low degree of antibiotic resistance in *Staphylococcus aureus* strains isolated from milk samples collected from cows with clinical and subclinical mastitis. Resistance to one or two classes of antibiotics was observed in 24.1% of strains, and a single strain showed multiple resistance to a wide range of antibiotics. Our results indicate resistance of the strains to several classes of antibiotics. None of the isolates from clinical mastitis showed complete resistance to ciprofloxacin. The diameter of the inhibition zone was between 12-17 mm (accoding to CLSI ≥21 mm sensible; 16-20 mm intermediate resistant and ≤15 mm resistant). Intermediate resistant pathogens cannot be considered susceptible to the antibiotic tested (Ali et al., 2010). The results of the study conducted by Saini coworkers (2012) revealed the usefulness of monitoring the use of antibiotics and their resistance patterns, in order to be able to manage the antibiotic resistance. It can thus be deduced that the antibiotics with which there is an increased resistance are those often used for the treatment of different diseases in cattle, thus determining the resistance of the strains carried by these animals. The evaluation of the antibiotic resistance pattern in correlation with the epidemiological investigation can guide the therapy in order to obtain the expected results. Of the strains resistant to more than one antibiotic, most were resistant to the combinations of amoxicillin/clavulanic acid, neomycin, streptomycin ad cefoperazone. A MAR index value ≥0.2 was observed in 100% of the resistant pathogens.

Table 1. The antimicrobial sensitivity patterns of the isolated strains

Nr.crt	AMC	P	N	S	AX	CEP	CIP	OT
S1	I	I	R	I	I	R	I	I
S2	R	I	R	R	I	R	I	I
S3	R	I	I	R	I	R	I	R
S4	R	I	I	I	I	I	I	I
S5	R	I	R	R	I	I	I	R
S6	R	I	I	R	I	R	I	I
S7	R	R	R	I	I	R	I	I
S8	R	R	I	R	I	R	I	I
S9	R	R	R	R	I	R	I	R
S10	R	8	I	R	I	R	I	I
S11	R	R	I	R	I	R	I	I
S12	R	I	R	R	I	R	I	I
S13	R	I	I	R	I	I	I	I
S14	R	I	I	R	I	I	I	I
S15	R	I	R	R	R	I	I	I
S16	R	I	R	R	R	I	I	I

Legend: I – intermediate resistant, R- resistant, S1-S4 *Staphylococcus xylosus,* S5-S7 *Staphylococcus lentus,* S8, *Shigella spp.* S9 *Actinobacter bauman,* S10 *Cryseomonas luteola,* S11-S15 *Enterobacter cloacae,* S16 *Echericia vulneris* (7.69%)
AMC - amoxicillin/clavulanic acid, AX– amoxicillin, S- streptomycin, CFP-cefoperazone, CIP- ciprofloxacin, OT-oxytetracycline, P-penicillin, N-neomycin

Table 3. Multiple antibiotic resistance (MAR) index of antibiotics against isolated

Drug	Total number of rezistent strains	MAR index of the tested antibiotics
Amoxicillin+Clavulanic acid	16	1
Penicillin	16	1
Neomycin	16	1
Streptomycin	16	1
Amoxicillin	16	1
Cefoperazone	16	1
Ciprofoxacin	16	1
Oxytetracyclin	16	1

Studies performed in the Louisiana, USA, in order to determine the prevalence of mastitis in dairy cows that have reached sexual maturity, showed that intra-mammary infection were present in 97% of animals and 75% of quarters. *Staphylococcus hyicus, Staphylococcus aureus* and *Staphylococcus chromogenes* were the most common isolated bacterial strains (Nickerson, 2009). About 28% of the animals and 16% of the quarters had clinical mastitis. Other researchers indicated the importance of *Staphylococcus aureus* in mamamry gland infections in dairy cows. Both lactating and dry cows are included in the *Staphylococcus aureus* infection control program. The number of cases of mastitis reported to be cured following *Staphylococcus aureus* infection varies considerably (Barkema et al., 2006.

From an economic point of view, the choice of treatment protocol must be fully justified. It is important to carefully select antibiotics, as well as the correct administration and compliance with waiting periods. One of the biggest public health problems in the world is antibiotic resistance. Due to improper treatment, antimicrobial-resistant microorganisms can spread very easily in nature. Treatment failures can occur due to resistance to effective broad-spectrum antibiotics (Ali et al., 2010). These treatment failures have a major impact on animal welfare and also lead to significant economic losses.

CONCLUSIONS

The varied and potentially pathogenic microflora isolated from cows with subclinical mastitis may have implications for the production of gastrointestinal disorders in dairy products consumers. In addition, the antibiotic resistance present in these bacterial strains may play a role in spreading to other animals and humans. Isolated species may indicate fecal pollution (*Shigella* spp., *Escherichia vulneris,*

Enterobacter cloacae) suggesting the need for better shelter hygiene. as well as the mammary gland before milking. Total or partial resistance (resistant colonies) to several antibiotics is worrying, suggesting the harmless use of antibiotics in the territory either to control possible clinical mastitis or to treat conditions other than mastitis in individuals in the experimental group. The treatment must be individualized according to the bacterial species and its degree of resistance according to the antibiogram, at the same time as the application of non-specific measures for the prophylaxis of mastitis.

REFERENCES

Ali, S. Q., Zehra, A., Naqvi, B. S., Shah, S., & Bushra, R. (2010). Resistance pattern of ciprofloxacin against different pathogens. *Oman medical journal*, *25*(4), 294–298. doi: 10.5001/omj.2010.85

Barkema H.W., Schukken Y.H., Zadoks R.N. (2006) Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J Dairy Sci*. 89(6):1877-95. DOI: 10.3168/jds.S0022-0302(06) 72256-1

Belay, N., Mohammed, N., & Seyoum, W. (2022). Bovine Mastitis: Prevalence, Risk Factors, and Bacterial Pathogens Isolated in Lactating Cows in Gamo Zone, Southern Ethiopia. *Veterinary medicine (Auckland, N.Z.)*, *13*, 9–19. https://doi.org/10.2147/VMRR.S344024.

Burvenich C., Bannerman D.D., Lippolis J.D., Peelman L., Nonnecke B.J., Kehrli M.E., Paape M.J. (2007) Cumulative physiological events influence the inflammatory response of the bovine udder to *Escherichia coli* infections during the transition period. *J Dairy Sci*. 90 (Suppl 1):E39-54. doi: 10.3168/jds.2006-696.

Cheng, W. N., & Han, S. G. (2020). Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments - A review. *Asian-Australasian journal of animal sciences*, *33*(11), 1699–1713. doi: 10.5713/ajas.20.0156

Dworecka-Kaszak, B., Krutkiewicz, A., Szopa, D., Kleczkowski, M., & Biegańska, M. (2012). High prevalence of *Candida* yeast in milk samples from cows suffering from mastitis in Poland. *The Scientific World Journal*, 196347. doi: 10.1100/2012/196347

Lakew, B., Fayera, T. & Ali, Y. (2019) Risk factors for bovine mastitis with the isolation and identification of *Streptococcus agalactiae* from farms in and around Haramaya district, eastern Ethiopia. *Trop Anim Health Prod*. 51, 1507–1513. doi: 10.1007/s11250-019-01838-w

Nickerson S.C. (2009) Control of heifer mastitis: antimicrobial treatment-an overview. *Vet Microbiol*. 134(1-2), 128-35. doi: 10.1016/j.vetmic.2008.09.019

Oliveira L., Langoni H. & Ruegg P.L. (2012) Minimum inhibitory concentrations of *Staphylococcus aureus* revovered from clinica land subclinical cases of bovine mastitis. *J.Dairy Sci*. 95,1913-1920 doi: 10.3168/jds.2011-4938

Radsak, M., Iking-Konert, C., Stegmaier, S., Andrassy, K., & Hänsch, G. M. (2000). Polymorphonuclear neutrophils as accessory cells for T-cell activation: major histocompatibility complex class II restricted antigen-dependent induction of T-cell proliferation. *Immunology*, *101*(4), 521–530. https://doi.org/10.1046/j.1365-2567.2000.00140.x

Thorberg B.M., Danielsson-Tham M.L., Emanuelson U., Persson Waller K. (2009) Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *J Dairy Sci.* 92(10):4962-70. https://doi.org/10.3168/jds.2009-2184

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