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COMPARISON OF CULTIVATION TESTS TO DETECTION MASTITIS IN DAIRY COWS

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ABSTRACT

Mastitis is an inflammation of the mammary gland which has a particular importance in ruminants. The purpose of this study was to evaluate and compare two methods for cultivation of udder pathogens: classical laboratory cultivation on Columbia agar with 5% sheep blood versus MicroMast rapid plates. The results allowed assessing the incidence of mastitis and the prevalence of the pathogens. During the study have been investigated 227 cows in a dairy farm localized in the east of Slovakia. Subsequently, 141 quarter milk samples from the positive cows with California mastitis test score 1-4 have been undergone the laboratory culture on Columbia agar and MicroMast test in accordance with their respective steps. The values obtained from these tests showing sensitivity of positive samples using the MicroMast test at the level of 64.5%, and sensitivity of cultures on Columbia blood agar at the level of 61.7%. After biochemical identification of cultured isolates, the Columbia blood agar and MicroMast test identified both as the main pathogen present *Staphylococcus aureus (S. aureus)* with results of 13.4% and 14.9%, respectively. On the base of results, both tests are comparable and therefore a test that speeds up testing is more useful in practice. It will be a relevant importance in the following decades to succeed in the development of tests with directly detection of udder pathogens.

Keywords: Cows, Detection, Mastitis, Cultivation, MicroMast test, Udder pathogens

INTRODUCTION

Milk from ruminants is among the most widely used animal products globally due to its specific composition and nutritional value. Approximately 150 million farmers around the world are involved in its production, catering to over 6 billion people. Obtained milk is a traditional raw material to produce a wide range of dairy products, which are unique in their composition. However, EU regulations emphasize that such products must originate from healthy animals (**Audarya et al., 2022; FAO, 2022**).

Currently, mastitis, an inflammation of the mammary gland, is one of the most significant and challenging diseases in dairy cattle. Its occurrence has a substantial impact on the economy of production farms, despite improvements in milk production hygiene and zootechnical control. Mastitis affects health and utility parameters such as decreased milk production, increased somatic cell count (SCC; directly affecting milk price), reduced qualitative components (lactose and casein, essential for cheese processing), and also increases undesirable elements (lipase and plasmin, affecting milk longevity). Additionally, indirect losses caused by mastitis include increased culling of diseased cows, loss of premiums, premature drying off, animal welfare aspects, and other related health issues. For clinical forms of mastitis, treatment costs can reach up to 200 EUR per case (**Cobirka et al., 2020**).

To date, more than 135 different causative agents of intramammary infection (IMI) in ruminants are recorded, including bacteria, viruses, yeasts, and algae. Up to 95% of IMIs are most caused by bacteria (**Tančin, & Uhrinčať, 2014**). Due to the polyetiological nature of mastitis, this disease cannot be completely eradicated from livestock, especially due to environmental pathogens such as staphylococci, streptococci, mycoplasmas, or *Escherichia coli (E. coli)*, which attack the mammary gland from the surroundings. The disease can have clinical manifestations with apparent changes in milk appearance (results of inflammatory response) and signs of mammary gland inflammation, but it can also be subclinical, meaning that the infection is present (and can spread to other animals) but without visible clinical symptoms (**Foltys, & Kirchnerova, 2003**).

Diagnostic tests for the detection of mastitis are divided into indirect, by which we determine SCC in milk in cases of subclinical mastitis, and direct so-called culture methods serving to identify the causative agents of inflammation of udder in all forms of mastitis. According to the place of use, we divide them into farm and laboratory methods. Laboratory diagnostics consists of bacteriological, cytological, biochemical examination and laboratory tests to determine the number of cellular elements in milk. Milk samples from individual dairy cows (quarter, half, mixed) or average samples obtained from dairy cow groups (cisterns, pools) are examined **(Škarda, & Škardová, 2000).** It is carried out on blood agar or on special agars. Various identification tests based on DNA analysis can be used to characterize pathogens at different phylogenetic levels. These methods can detect either DNA or RNA.

Currently, mastitis can be detected using on-farm tests, with the most common being the California Mastitis Test (CMT), but it doesn't allow pathogen identification. The presence of udder pathogens is most frequently detected in the laboratory through cultivation on various selective agars, which is time-consuming. Lately, the goal is to introduce tests that will be as effective as laboratory cultivation and can be performed by a trained person directly on the farm, saving farmers time associated with sample transportation and evaluation (**Prášek, 2023**).

The aim of the study was to compare two tests for the cultivation of udder pathogens from samples of raw cow's milk using classical laboratory cultivation on Columbia agar with 5% addition of sheep's blood and the MicroMast plates.

MATERIAL AND METHODS

Dairy Cow Production, Housing, and Milking

For the practical purposes of the study, a farm of 270 Slovak Spotted Cattle was monitored. The farm was in eastern Slovakia. The farming environment consisted of a modern building with high air ventilation and automated waste removal. The cows were housed in a stable divided into 2 sections using a feeding table, with

four separate subsections within each. Each subsection had 42 cubicles arranged in three rows facing each other.
During the study, 227 dairy cows were milked twice a day at 5:00 AM and 4:00 PM in a parallel milking parlour 2 × 12 (BouMatic). Before milking, milk from each quarter of the udder underwent sensory analysis in a cup with double bottom. Teat disinfection was carried out using a foaming preparation G-Mix Power containing chlorine dioxide (Agromont, Nitra, Slovakia), followed by drying with a disposable paper towel. After automated milking was completed with a milk flow rate lower than 0.2L/min, final teat disinfection was performed using Power Blue Mix (Agromont, Nitra, Slovakia), a product based on lactic acid.

Examination of Cows with Milk Sampling

For the purpose of milk sampling, out of the 227 cows, each cow was thoroughly examined based on clinical examination and udder palpation. Milk forestrips from each quarter were subjected to sensory examination and evaluated using the California Mastitis Test (CMT) according to **Tančin et al. (2006)**. For the laboratory diagnosis of bacterial pathogens, based on the **National Mastitis Council (2001)** guidelines, on the base of positive CMT scores ranging from 1 to 4, the 141 quarter milk samples were examined (Table 1). The same samples were used for the cultivation of udder pathogens on MicroMast plates (Prášek, CZ) and on plates with Columbia agar supplemented with 5% sheep blood (Oxoid, Ltd, UK). All samples were transported to the laboratory and examined on the same day.

Table 1 Evaluation of milk quarter samples from 227 dairy cows and interpretation of CMT score

CMT score	SCC	Interpretation	Investigated quarters (n = 908)	
			(%)	**n**
N (negative)	0 - 200,000	Healthy quarter	*80.0*	726
T (trace)	200,000 - 400,000 (±50,000)	Latent mastitis*	*2.5*	23
1	400,000 - 650,000 (±150,000)	Subclinical mastitis*	*6.1*	55
2	850 000 - 1,200,000 (±200,000)	Subclinical mastitis Serious mastitis*	*3.0*	27
3	1,500,000 - 5,000,000 (±300,000)	Serious mastitis	*4.5*	41
4	Over 5,500,000	Serious mastitis	*2.0*	18

Legend: CMT - The Californian Mastitis Test, SCC - somatic cell count, Latent mastitis* - milk appears normal, but infection is still present in samples of raw milk without changing of SCC with negative CMT score, Subclinical mastitis* - no signs are observed, the udder and milk appears normal, but infection is still present with positive CMT score and increased SCC. Serious* or clinical mastitis – signs range from mild to severe with positive CMT score, bacteriological cultivation, high level of SCC, changing the consistency of the milk with the presence of flakes, clots or pus and reduction or loss of milk production with clinical signs.

Cultivation of Samples and Detection of Udder Pathogens

An inoculum of each milk sample (0.2 ml) was streaked onto plates with Columbia agar supplemented with 5% sheep blood (Oxoid Ltd, UK), which were incubated at 37 °C and evaluated after 24 hours. For further identification of bacterial pathogens causing mastitis, colonies from the blood agar were subcultured based on morphological characteristics onto different selective culture media: Edwards Medium, Staphylococcal Medium No. 110, and MacConkey Agar (Oxoid, Ltd., Basingstoke, Hants, UK). The subcultures were incubated for an additional 24 hours at 37 °C, and tests for catalase activity, haemolysis, pigment production, coagulase, and Gram staining were performed according to **Malinowski et al (2006)**. Identification of each species was conducted using biochemical tests: STAPHYtest 24, STREPTOtest 24, or ENTEROtest 24, and evaluated using the TNW ProAuto 7.0 program (Erba-Lachema, Brno, CZ) with a species identification probability exceeding 90%.

MicroMast Rapid Test

This test is designed for rapid 24-hour cultivation of samples directly under on-farm conditions. It utilizes a kit for milk sample collection, culture plates, and a portable farm incubator. Quarter milk samples collected from positive cows were cultivated on MicroMast plates at the same time, on the same day, at a temperature of 37 °C. Results were analysed after 24 hours for each plate, which is divided into three zones.
In the first zone 'A,' contamination of samples is ruled out as all samples are negative without any growth present. The second zone 'B' is designated for the identification of Gram-positive pathogens, such as *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus agalactiae*, *Streptococcus uberis*, other environmental streptococci, *Enterococcus* spp., *Corynebacterium* spp., *Trueperella pyogenes*, or *Bacillus* spp. The third zone 'C' is intended for the identification of Gram-negative pathogens, including *E. coli*, *Klebsiella* spp., other *Enterobacteriaceae*, *Proteus vulgaris*, *Pseudomonas* spp., *Serratia marcescens*, or *Pasteurella* spp. Subsequently, after cultivation on MicroMast plates, colonies forming units (CFUs) were analysed using additional tests and subcultured on selective media in the same manner as in the previous procedure for analysing bacteria pathogens from Columbia blood agar.

RESULTS AND DISCUSSION

Mastitis is considered a global disease that manifests in various forms (subclinical, chronic, and clinical), mainly caused by contagious and environmental bacteria. One of the first indicators of udder infection is an increased somatic cell count (SCC), often associated with decreased milk production (**Tongeľ, & Mihina, 2005**). Somatic cells remain a valuable tool for initial milk assessment, as differences between healthy and diseased animals are evident. The California Mastitis Test (CMT) is commonly used on farms for rapid assessment of quarter milk samples, particularly for detecting subclinical mastitis forms (**Tančin, & Tančinová, 2008**).
During the comprehensive udder examination, the CMT was performed on the first foremilk samples from each of the 227 dairy cows. Out of 908 examined quarters, 18 were atrophied or lacked milk production. Among the evaluated quarters, 726 (80.0%) were negative with a CMT score of 0. From the 141 (15.6%) examined quarters with a positive CMT were highest proportion with a score 1 and 3 (Tab. 1). In addition to assessing SCC, new methods for mastitis detection are being sought, with the development of new tests to enable farmers to respond to positive cases promptly. This involves implementing appropriate measures or initiating early treatment, which tends to be more effective and cost-efficient compared to later stages, where pathogens develop resistance to intramammary antibiotics. Currently, the development of rapid on-farm sample testing methods is a solution, reducing the time and costs associated with sample transport and laboratory detection (**Prášek, 2023**).
To compare two cultivation methods, individual milk samples were taken from 141 positive quarters with CMT scores of 1-4. These samples underwent laboratory diagnostics on Columbia agar with 5% sheep blood and were simultaneously streaked onto MicroMast plates. After 24 hours of cultivation on blood agar, bacterial udder pathogens were confirmed in 87 samples (61.7%). In 54 cases (38.3%), samples were negative without colony growth on the cultures (Table 2).
Using the same procedure, after cultivation on MicroMast plates, 91 positive samples (64.5%) were recorded in sectors B and C, designed for differentiation between Gram-positive and Gram-negative bacteria. No growth was observed in sector A on any plate, indicating uncontaminated samples. The remaining 50 samples (35.5%) were negative without growth in all three sectors (Table 3). For further analysis of bacterial pathogens, all cultures from a subculture on blood agar and MicroMast plates were subjected to selective media cultivation, with identification using biochemical tests. In both cases, coagulase-negative staphylococci (CNS) and *Staphylococcus aureus* were most frequently isolated. *Escherichia coli* was isolated in both cases among other pathogens (Table 2 and 3).

Table 2 Cultivation of pathogens on Columbia agar with 5% sheep blood and their representation after identification by biochemical tests

Status / pathogen	Number of samples from tested quarters	% (from 141 quarter milk samples)
Negative	54	38.3
Positive	87	61.7
Total	141	100.0
***Staphylococcus* spp.**		
S. aureus	19	13.5
*S. warneri**	9	6.4
*S. chromogenes**	8	5.7
*S. epidermidis**	4	2.8
S. intermedius	3	2.1
*S. hyicus**	2	1.4
*S. sciuri**	1	0.7
*S. cohni urealytic**	1	0.7
Other pathogens		
E. coli	22	15.6
Proteus spp.	7	4.9
E. faecalis	3	2.1
Mixed IMI infection**	8	5.7

Note: CNS* – coagulase negative staphylococci; Mixed IMI infection** - mixed intramammary infection represent a positive sample with two or more udder pathogens. *Staphylococcus intermedius*, *Enterococcus faecalis*

Table 3 Evaluation of 141 quarter milk samples using the MicroMast test

	No. of quarters	%	Positive in "C" n / %	Positive in "B" n / %
Positive	91	64.5	28 (19.9%)	63 (44.7%)
Negative	50	35.5	-	-
Representation of bacterial pathogens after biochemical identification from MicroMast plates				
	Positive in "C"	%	Positive "B"	%
E. coli	20	14.2		
Proteus spp.	3	2.1		
S. epidermis *			3	2,1
S. aureus			21	14.9
*S. sciuri**			1	0.7
*S. warneri**			11	7.8
S. intermedius			3	2.1
*S. felis**			2	1.4
*S. chromogenes**			17	12.1
Mixed IMI infection**	5	3.5	5	3.5

Note: The C zone is part of the test enabling the detection of gram-negative pathogens; The B zone is part of the test enabling the detection of gram-positive pathogens; CNS* – coagulase negative staphylococci; Mixed IMI infection** - mixed intramammary infection represent a positive sample with two or more udder pathogens. *Staphylococcus intermedius*

According to **Hogeveen et al. (2021)** and **Woudstra et al. (2023)**, udder mastitis presents a significant health issue due to the wide range of causative agents. **Holko et al. (2019)** found in their study that many cases of intramammary infections (IMIs) in Slovak dairy herds are caused by Gram-positive microorganisms such as *Staphylococcus* spp. or *Streptococcus* spp., which was also confirmed in our study. However, the prevalence of IMIs caused by coliform bacteria occurs up to 20.0%, depending on the farm structure and hygienic conditions. Our results show that 41% and 15% of IMIs were caused by staphylococci and *Escherichia coli*, respectively. Of the coagulase-negative staphylococci, *Staphylococcus warneri, Staphylococcus chromogenes* and *Staphylococcus epidermidis* were confirmed in both tests after biochemical identification from positive milk samples. Despite the accepted role of these bacteria as frequent pathogens causing mastitis in cows, the pathogenicity of the different CNS (coagulase negative staphylococci) species varies widely (**Sameer et al., 2018**). In the studies conducted by **Sawant et al. (2009)** and **Thorberg *et al*. (2009)** were CNS the most prevalent pathogens causing subclinical mastitis in dairy cows and ewes. Although less pathogenic than *S. aureus*, CNS can also produce persistent subclinical or clinical mastitis. After infection of CNS is significantly increased SCC, CMT, cause clinical mastitis as well as producing thermostable enterotoxins.

Table 4 Comparison of pathogens based on cultivation on Columbia agar with 5% sheep blood and MicroMast plates.

Pathogen	Columbia blood agar		MicroMast plates	
	Positive	% (from141 samples)	Positive	% (from141 samples)
***Staphylococcus* spp.**				
S. aureus	19	*13.4*	21	*14.9*
*S. chromogenes**	8	*5.7*	17	*12.1*
*S. warneri**	9	*6.4*	11	*7.8*
*S. epidermidis**	4	*2.8*	3	*2.1*
S. intermedius	3	*2.1*	3	*2.1*
*S. hyicus**	2	*1.4*	-	-
*S. felis**	-	-	2	*1.4*
*S. sciuri**	1	*0.7*	1	*0.7*
*S. cohni urealyticus**	1	*0.7*	-	-
Other pathogens				
E. coli	22	*15.6*	20	*14.2*
Proteus spp.	7	*5.0*	3	*2.1*
E. faecalis	3	*2.1*	-	-
Mixed IMI infection**	8	*5.7*	10	*7.1*
Total	**87**	***61.7***	**91**	***64.5***

Note: CNS* – coagulase negative staphylococci; Mixed IMI infection** - mixed intramammary infection represent a positive sample with two or more udder pathogens. *Staphylococcus intermedius, Enterococcus faecalis*

Thorberg et al. (2009) confirmed and demonstrated one or two types of *S. epidermidis* (*Staphylococcus epidermidis)* in two monitored herds of dairy cows. The dominant types of *S. epidermidis* from milk were also isolated from skin of the people who were responsible for milking cows because isolation of *S. epidermidis* from human skin is more common than isolation from bovine skin. The authors conclude that humans who are daily in contact with animals are probably the main source of infection for cows.

Other types of bacteria in our study were represented by *Streptococcus agalactiae*, *Trueperella pyogenes*, *E. coli*, *Enterococcus* spp. and *Bacillus* spp. during monitored period. The importance of using the results of diagnostics is manifested even in control of cows, and its inclusion into the herd rearing. Identification of bacterial udder pathogens through biochemical tests and their comparison from both methods revealed differing counts, particularly for coagulase-negative staphylococci and *Proteus* spp. There was a relatively similar concurrence for *S. aureus* and *E. coli*, which were the most prevalent among the tested samples in both methods (Table 4). When comparing the MicroMast test with laboratory diagnostics using cultivation on Columbia agar supplemented with 5% sheep blood, a higher sensitivity of positive samples was observed with the MicroMast test at a level of 64.5%, compared to cultivation on blood agar at a level of 61.7% (Table 1).

CONCLUSION

The data used in this study allowed us to demonstrate that although the CMT does not enable pathogen identification, it can indicate ongoing or incipient udder inflammation. By comparing two methods of milk sample cultivation, the sensitivity of positive samples was observed with the MicroMast test at a level of 64.5%, by cultivation on blood agar at a level of 61.7%. In both cases, CNS and *E. coli* were the most frequently isolated pathogens. Among contagious pathogens, *S. aureus* was isolated at a level of 13.4% Columbia agar with 5% sheep blood and in 14.9% after cultivation on MicroMast plates, followed by identification through biochemical tests. The confirmation of the presence of *Staphylococcus* spp. and a significant proportion of coagulase-negative staphylococci in milk samples from mastitic cows highlights potential health risks for consumers. Therefore, it is necessary for farmers to be able to identify infectious animals as soon as possible. The use of the CMT complemented with rapid cultivation tests is one of the ways farmers can directly capture subclinical infections caused by udder pathogens on the farm, thus initiating early treatment and reducing the health and economic impact of mastitis in cows. Based on the results of comparing the sensitivity of both used methods, we can conclude that MicroMast as a method for early detection of mastitis is comparable and therefore applicable in practice.

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