Cultivation tests for rapid detection of mastitis pathogens in dairy cows

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Abstract

The aim of this study was to evaluate and compare two methods for rapid detection of udder pathogens: MicroMast™ rapid plates versus ClearMilk Test. Both methods belong to cultivation laboratory methods for the detection of Gram-positive (staphylococci and streptococci), and Gram-negative bacteria directly under on-farm conditions. During the study, 520 cows were examined on dairy farms localized in the east of Slovakia. Subsequently, 144-quarter milk samples from the positive cows with California mastitis test scores 1–3 underwent laboratory cultivation on both rapid tests. Values obtained from these tests showed the sensitivity of positive samples using the MicroMast test at the level of 84.7% and the sensitivity of the ClearMilk test at the level of 93.1%. After biochemical and protein identification of cultured isolates, the main pathogens present on both rapid tests MicroMast and ClearMilk were identified as *Staphylococcus* spp. (*S. aureus* and *S. chromogenes*) and *Streptococcus* spp. (*Str. bovis*). Based on the results, both tests are comparable and therefore they can potentially be used in practice for rapid detection of udder pathogens.

Cattle, udder health, MicroMast test, ClearMilk test

Mastitis is a multi-aetiological disease associated with the cattle production system and rearing environment. It is manifested by physical, chemical, and bacteriological changes in the mammary gland tissue (Lipkens et al. 2019). Mastitis is a result of inflammation of the udder gland tissue and the defense reaction of the organism to some factors, mainly the microorganisms present inside the mammary gland (Bortolami et al. 2015).

The aetiology of cattle mastitis can be infectious or non-infectious. Cell-walled bacteria are the most commonly reported mastitis-causative agents, although a variety of other microorganisms, such as mycoplasmas, chlamydia, algae, fungi and viruses, have also been associated with the disease (Bradley 2002; Morales-Ubaldo et al. 2023).

The aetiological agents include a variety of Gram-positive and Gram-negative bacteria and can be either contagious (e.g., *Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma spp.*) or environmental (e.g., *Escherichia coli, Enterococcus* spp., coagulase-negative staphylococci (CNS), *Streptococcus uberis*) (Cheng and Han 2020). The most prevalent form of the disease is subclinical mastitis, which leads, in the absence of clinical signs, to decreased milk production, increased somatic cell count (SCC), and an increased risk of clinical mastitis during lactation (Tomassoni et al. 2023).

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

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control. Mastitis is responsible for approximately 60–70% of all antimicrobials administered on dairy farms (Cobirka et al. 2020). On average, the total cost of bovine mastitis is $147 (Hogeveen et al. 2019). For clinical mastitis, losses range from $179 to $518 (Bar et al. 2008; Rollin et al. 2015). In subclinical forms, there are no sensory changes in milk, but their frequency is much higher (15–40 times) compared to clinical ones (Bergonier et al. 2003).

The correct management of mastitis is based both on preventive and treatment action. With the increasing concern for antimicrobial resistance, it is strongly recommended to treat only the mammary quarters presenting intramammary infection. For this reason, a timely and accurate diagnosis is fundamental. The possibility of detecting and characterizing mastitis directly on the farm would be very useful in choosing the correct management protocol. Some on-field diagnostic tools are already routinely applied to detect mastitis, such as the California Mastitis Test (Tomassoni et al. 2023).

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 the udder in all forms of mastitis. According to the place of use, we divide them into farm and laboratory methods. Laboratory diagnostics consist of bacteriological, cytological, and biochemical examinations. Milk samples from individual dairy cows (quarter, half, mixed) or average samples obtained from dairy cow groups (cisterns, pools) are examined (Skarda and Skardova 2000). It is carried out on blood agar or 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.

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 (Prasek et al. 2010). The literature indicates that while there are many diagnostic methods for mastitis, there is still a need to develop new tools. Further research is necessary to establish objective detection parameters and prognostic indicators. It is desirable to implement tests that demonstrate both high sensitivity and high specificity (Stanek et al. 2024).

The objective of this study was to evaluate and compare two methods for rapid detection of udder pathogens: MicroMast rapid plates versus ClearMilk Test. Both methods belong to culture laboratory methods for the detection of Gram-positive (staphylococci and streptococci), and Gram-negative bacteria directly in farm conditions.

Materials and Methods

For this study, the clinical examination of the cows and the collection of milk samples were approved by the Ethics Committee at the University of Veterinary Medicine and Pharmacy in Kosice no. EKVP 2022/05 following EU legislation 2010/63/EU, article 1:5 (practices not likely to cause pain, suffering, distress, or lasting harm equivalent to or higher than, that caused by the introduction of a need to follow good veterinary practice).

Dairy cow production, housing, and milking

During the study, 520 dairy cows were examined on dairy farms of Holstein Cattle breed localized in the east of Slovakia. The dairy cows examined on both farms were kept in a free housing system on straw litter, with *ad libitum* access to water. They were fed a mixed feed based on silage, hay, and concentrate, in agreement with the nutritional requirements of dairy cattle (NMC 2001). The exact amount of feed was determined by lactation performance, and the rations met the nutritional requirements of high-producing cows. The average milk yield was 9,000 kg per lactation. Cows were milked twice daily in parallel milking parlours 2 × 12 (BouMatic, Skjern, Denmark), or herringbone (DeLaval, Cardiff, UK) parlours. Milk samples from dairy cows were taken during milking (samples from the CMT positive quarters of the udder).

Examination of cows with milk sampling

The study involved 520 lactating cows, and udder health evaluation included clinical examination, sensory analysis of milk from fore stripping of each udder quarter, followed by assessment of the California mastitis test (CMT) (Indirect Diagnostic Test, Krause, Denmark).

Clinical changes in the udder were diagnosed by the presence of observable signs of inflammation in the infected quarter, such as swelling, heat, pain, or redness, and by the presence of clots and flakes in the milk, or by its abnormal colour or consistency. The CMT was performed according to Jackson and Cockroft (2002), using equal volumes of milk and alkyl-aryl-sulphonate, and was evaluated as: 0) negative (−) with SCC 0–200,000/ml, not infected, healthy quarter, no thickening of the mixture; 1) trace (±) with SCC 200,000–400,000/ml, possible infection, slight thickening of the mixture; 2) weak positive (+) with SCC 400,000–650,000/ml, infected, distinct thickening of the mixture but no tendency to form a gel; 3) positive (++) with SCC over 850,000/ml infected, distinct thickening of the mixture with tendency to form a gel.

From each udder quarter with positive CMT result (ranging from 1 to 3), we took a milk sample for bacteriological cultivation and a sample for determining the number of somatic cells (144 milk samples). We determined the number of somatic cells on the Somacount 150 device (Bentley Instruments, Inc., Chaska, Minnesota, USA). The same samples were used for the cultivation of udder pathogens on MicroMast™ plates (Prasek, Svetla nad Sazavou, Czech Republic), and ClearMilk test (LabMediaServis s.r.o, Jaromer, Czech Republic). Milk was collected aseptically from teats directly into sterile 10 ml tubes, holding the tubes at an angle of approximately 45 °C. The samples were kept at 4–8 °C during transportation to the laboratory.

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 applied (0.05 ml), and cultivated on MicroMast plates at the same time on the same day, at a temperature of 37 °C. Results were analysed after 24 h for each plate, which is divided into three zones (Plate II, Fig. 1).

Growth in the first zone 'A' is used to exclude sample contamination and as a confirmation of growth (non-selective agar). The second zone 'B' is designated for the identification of Gram-positive pathogens, such as *Staphylococcus aureus*, 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.

ClearMilk test

This test is used for rapid 24-h cultivation of milk samples using on-farm conditions. A simple reading of results based on different colours of grown colonies can be used for the rapid screening of dairy cows excreting contagious or environmental pathogens in milk as part of anti-mastitis programmes. The milk samples were applied (0.05 ml) to the surface of the plate with three different selective chromogenic agars. ClearMilk test was incubated for 22–26 h at a temperature of 37.5 °C. Results were analysed after the incubation of each plate, which is divided into three zones (Fig. 1):

Sector G−: colour-coded bacteria grow here, which belong to the group of so-called Gram-negative rods. These include two important causative agents of dangerous mastitis: *Escherichia coli* and *Klebsiella pneumoniae* ssp. *pneumoniae.*

Staph sector: bacteria of the genus *Staphylococcus* spp. grow here, as well as some others, e.g. *Staphylococcus aureus.*

Strept sector: bacteria of the genus *Streptococcus* spp. grow here, as well as some others. These include important causative agents of mastitis, e.g. *Streptococcus uberis, S. agalactiae, S. parauberis*, and *S. dysgalactiae*.

Cultivation of samples and detection of udder pathogens

For more exact identification of bacterial pathogens causing mastitis, colonies forming units (CFUs) from both tested cultivation plates 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) in additional aerobic conditions for 24 h at 37 °C. The 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%.

The MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) device was used to definitively identify bacteria by Becker and Schubert (2020). A milk sample was classified positive for contagious pathogens (*S. aureus, Streptococcus agalactiae*) if the presence of at least one colony-forming units (CFU/ml) was detected, and for other pathogens the sample was marked positive if at least five colony-forming units were identified. A sample was classified as contaminated if three or more types of pathogens were detected, none of which were contagious species, and mixed growth was detected simultaneously in more sectors. Results which provided Maldiscore values [log(score)] in the range of 2.300–3.000, were indicated as highly probable species identification.

Results

After the inspection and palpation of the udder, a CMT was performed on the first foremilk samples from each of the 520 dairy cows. Of the 2,080 examined quarters, 49 were atrophied or without milk production. Of the 2,031 quarters evaluated, 1,887 (92.9%) were negative with a CMT score of 0. Positive samples with a CMT score of 1 to 3 in numbers 144 (7.09%) were tested for mammary gland pathogens using two culture methods.

The samples were simultaneously inoculated onto MicroMast and ClearMilk plates. After 24 h of culture on MicroMast plates, bacterial udder pathogens were confirmed in 122 samples (84.7%) in sectors B and C, intended for differentiation between Gram-positive (72.1%) and Gram-negative (27.9%) bacteria. With the simultaneous growth in sector A in all positive cases of growth in sectors B or C, contamination of the sample was excluded.

In 22 cases (15.3%), the samples were negative without colony growth in all sectors (Table 1).

Table 1. Evaluation of 144 quarter milk samples using MicroMast test.

	No. of quarters	%	Positive in 'C' n / %	Positive in 'B' n / %
Positive	122	84.7	34 / 27.9	88 / 72.1
Negative	22	15.3	-	-
Representation of bacterial pathogens after biochemical identification from MicroMast plates				
	Positive 'C'	%	Positive 'B'	%
E. coli	23	67.6		
Proteus spp.	11	32.4		
Bacillus spp.			6	6.8
S. aureus			23	26.1
S. intermedius			8	9.1
*S. sciuri**			2	2.3
*S. epidermidis**			7	7.9
*S. chromogenes**			22	25.0
Streptococcus bovis			16	18.3
Mixed IMI infection**	-	-	4	4.5

Zone 'C' is part of the test enabling the detection of Gram-negative bacteria. Zone 'B' zone is part of the test enabling the detection of Gram-positive bacteria.

*Coagulase negative staphylococci; **mixed intramammary infection represents a positive sample with three or more udder pathogens.

When evaluating growth on the ClearMilk test under the same culture conditions, positivity was recorded in 134 cases (93.1%). In the sector intended for the growth of Gram-positive bacteria from the strain *Streptococcus* spp., positive growth was detected in 25.4% cases, in the sector for *Staphylococcus* spp. the positivity rate was recorded in 61.2% of cases, and in the growth sector for Gram-negative bacteria growth was recorded in 13.4% of samples. In 10 negative samples (6.9%), colony growth was not observed in either sector (Table 2).

For further analysis of bacterial pathogens, all cultures from the subculture on the MicroMast and ClearMilk plates were subcultured on selective media with identification by biochemical tests. In both cases, CNS, *Staphylococcus aureus,* and *Streptococcus bovis* belonging to the Gram-positive bacteria were most often isolated. Of the Gram-negative, *Escherichia coli* bacteria were most often isolated.

Table 2. Evaluation of 144 quarter milk samples using ClearMilk test.

	No. of quarters	%	Positive 'Strept' G+	in %	Positive 'Staph' %	in G+	Positive G−	%
Negative	10	6.9	-	-	-	-	-	-
Positive	134	93.1	34	25.4	82	61.2	18	13.4
Representation of bacterial pathogens after biochemical identification from ClearMilk test								
Streptococcus spp.			5					
			14.7					
Streptococcus bovis			23					
			67.6					
S. aureus					24	29.3		
S. intermedius					6	7.3		
*S. epidermidis**					12	14.6		
*S. chromogenes**					26	31.7		
*S. sciuri**					10	12.2		
Other pathogens								
E. coli							18	100
Mixed IMI infection**			6	17.7	4	4.9		

Note: CNS* – coagulase negative staphylococci; Mixed IMI infection** - mixed intramammary infection represent a positive sample with three or more udder pathogens.

Table 3. Comparison of pathogens based on cultivation on MicroMast and ClearMilk tests.

Pathogen	MicroMast plates		ClearMilk Test	
	Positive	% (from 144 samples)	Positive	% (from 144 samples)
Staphylococcus spp.				
S. aureus	23	15.9	24	16.7
S. intermedius	8	5.5	6	4.1
*S. chromogenes**	22	15.3	26	18.1
*S. epidermidis**	7	4.9	12	8.3
*S. sciuri**	2	1.4	10	6.9
Streptococcus spp.	-	-	5	3.5
Streptococcus bovis	16	11.1	23	15.9
Other pathogens				
E. coli	23	15.9	18	12.5
Proteus spp.	11	7.6	-	-
Bacillus spp.	6	4.1	-	-
Mixed IMI infection**	4	6.9	10	2.8
Total	122	84.7	134	93.1

*Coagulase negative staphylococci; **mixed intramammary infection represents a positive sample with three or more udder pathogens.

Of the CNS, *Staphylococcus chromogenes* (15.3%, and 18.1%) was confirmed most frequently in both tests. Other CNS (*S. epidermidis, S. sciuri*) were isolated to a greater extent and subsequently identified from the ClearMilk test (8.3%, 6.9%). Other types of bacteria in our study were represented during the study period by *Streptococcus agalactiae, E. coli, Enterococcus* spp., and *Bacillus* spp.

Identification of bacterial udder pathogens by biochemical tests and the MALDI TOF MS method and their comparison with both rapid tests revealed similar numbers, especially for *S. aureus,* CNS, and *E. coli*. Differences, although non-significant, were observed for *Streptococcus bovis*, which was the most widespread representative of *Streptococcus* spp. in both methods (Table 3). When comparing the MicroMast and ClearMilk tests, a higher sensitivity of positive samples was observed in the ClearMilk test at 93.1% compared to cultivation on the MicroMast plate at 84.7% (Table 3).

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 (Halasa et al. 2007).

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, but it does not allow pathogen identification (Tancin and Tancinova 2008; Tomassoni et al. 2023). 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 (Prasek et al. 2010). According to Hogeveen et al. (2021) and Woudstra et al. (2023), udder mastitis poses a significant health problem due to the wide variety of causative agents.

In their study, Holko et al. (2019) found that many cases of intramammary infections (IMI) in Slovak dairy herds are caused by Gram-positive microorganisms such as *Staphylococcus* spp. or *Streptococcus* spp., which was also confirmed in our study. In the study of Hisira et al. (2020), the most prevalent bacteria were CNS (50%), followed by *Enterococcus* spp. (16.7%), *Proteus* spp. (11.1%), and *Aerococcus viridians* (11.1%). Similar results as in our study are described by Prasek et al. (2024), where the most frequent *Streptococcus uberis* was isolated in 16 (51.6%), CNS in 8 (25.8%), *E. coli* in 5 (16.1%) and no growth in 2 (6.5%) cases. The prevalence of IMI caused by coliforms reaches up to 20.0%, depending on farm structure and hygiene conditions (Holko et al. 2019). In our study, the prevalence of *E. coli* was 15.9% and 12.5%, respectively, but this does not reduce the severity of the infection, as it is still a relatively high percentage of incidence. Despite the accepted role of these bacteria as frequent pathogens causing mastitis in cows, the pathogenicity of different CNS species varies greatly (Sameer et al. 2018).

Saila et al. (2023) described the study with using the on-farm culture test (OFC; Mastatest HiSCC; Mastaplex Limited) which for detection of pathogens of subclinical mastitis uses a cartridge with 2 × 12 wells allowing 1 sample to be analyzed in duplicate (24 wells) or 2 samples analyzed simultaneously, each in 12 wells. Results of the milk analyses are reported hierarchically (*S. aureus* → CNS → other Gram-positive or coliform/ Gram-negative → no bacteria present) and emailed within 24 h. As determined by reference

standard and OFC test, about 45% of the 228 pooled milk samples contained *S. aureus* with the prevalence ranging from 20% to 84% across the 9 farms. The OFC test method reliably identifies the presence of *S. aureus* in quarter milk and pooled cow-level milk from cows with an SCC ≥ 150,000 cell/ml. In the absence of *S. aureus* in this milk, it reliably identifies CNS in pooled cow-level milk. Compared with the reference standard the method was rapid with results returned in 24 h of loading the cassette.

In studies conducted by Sawant et al. (2009) and Thorberg et al. (2009), CNS were the most prevalent pathogens causing subclinical mastitis in dairy cows and sheep. Although less pathogenic than *S. aureus*, CNS can also cause persistent subclinical or clinical mastitis. After CNS infection, SCC, and CMT increase significantly, causing clinical mastitis, as well as the production of thermostable enterotoxins. In our case, *S. chromogenes* was the most isolated CNS, detected by both culture tests. Thorberg et al. (2009) confirmed and demonstrated one or two types of *S. epidermidis* in two dairy herds studied. The dominant types of *S. epidermidis* from milk were also isolated from the skin of people who were responsible for milking cows since the isolation *of S. epidermidis* from human skin is more common than isolation from cowhide. The authors conclude that people who are in daily contact with animals are probably the main source of infection in cows. In milk samples in our experiment, *S. epidermidis* was the second most isolated CNS pathogen.

Only the results of a reliable on-farm test should be used to inform costly decisions (e.g., milk withhold, order of milking, culling of cows with recurring infections) and for selective use of antibiotics (McDougall et al. 2022; Saila et al. 2023). Thus, it is important to know the reliability of the test method (i.e., agreement with the reference standard, specificity, and sensitivity) in classifying a cow (or a quarter) as having IMI or not. Preferably, this is done by comparing the results of the on-farm test with a gold standard which is 100% accurate, but since no such gold standard exists for IMI, the comparison is made with the accepted reference standard while recognizing the limitations of this accepted standard (Dohoo et al. 2011). In the study by Ferreira et al. (2018) the milk samples were cultured using four on-farm plate systems, which constitute the most popular commercially available options in the USA: Accumast (FERA Animal Health LCC, Ithaca, NY), Minnesota Easy System Tri-Plate (University of Minnesota Laboratory for Udder Health, St. Paul, MN), Mastitis SSGN Quad plate (DQCI Services, Mounds View, MN) and Mastitis SSGNC Quad plate (DQCI Services, Mounds View, MN) by trained laboratory personnel. The on-farm culture test Accumast was the most accurate plate test for diagnosing mastitis-related pathogens, and a powerful tool to diagnose specific microorganisms, such as *E. coli* and bacteria belonging to *Streptococcus* spp. When diagnosis is based on Gram-positivity/Gram-negativity, Accumast remained the best test for all parameters with the exception of specificity (Ferreira et al. 2018).

The results of pathogen isolation using the MicroMast plates and ClearMilk rapid farm tests in our study were confirmed by two methods commonly used to isolate and diagnose udder pathogens in laboratory conditions. The incidence of individual pathogens, whether Gram-negative or Gram-positive, was comparable in our case using both tests.

In conclusion, the results obtained in this study allowed us to demonstrate that, even though the use of CMT in farm conditions does not allow the pathogen to be identified, it may indicate ongoing or incipient udder inflammation. By comparing two rapid methods of milk sample cultivation, the sensitivity of positive samples was observed at 84.7% on the MicroMast plates, and up to 93.1% when using the ClearMilk test. In both cases, CNS, *S. aureus, Streptococcus bovis,* and *E. coli* were the most isolated pathogens. Of the major pathogens*, S. aureus* was isolated at 15.9% on MicroMast plates, and 16.7% after culture on the ClearMilk test. The presence of *Streptococcus* spp. bacteria (3.5%), specifically *Streptococcus bovis* (15.9%) isolated and confirmed after culture in the ClearMilk test, also plays a significant role. The confirmation of the presence of *Staphylococcus* spp.,

Streptococcus spp., a significant proportion of CNS, and *E. coli* in milk samples of mastitic cows highlights the potential health risks for final consumers. It is therefore essential that farmers can identify infectious animals as early as possible. The use of CMT to detect the potential risk of mastitis, especially in cases of subclinical infections, supplemented by rapid culture tests to identify a particular group of pathogens, is one way of directly capturing infections caused by udder pathogens on the farm, thereby initiating early treatment and reducing the negative impact of mastitis on the health of cows and, consequently on the quality and safety of milk. Based on the results, both used tests are comparable, and therefore, they can be used in practice for a rapid detection of udder pathogens. However, microbiological identification of pathogens with the determination of an antibiogram remains an essential part of detection. The advantage of the ClearMilk test can be the more accurate detection and faster differentiation of Gram-positive pathogens thanks to separate zones for *Staphylococcus* spp. and *Streptococcus* spp.

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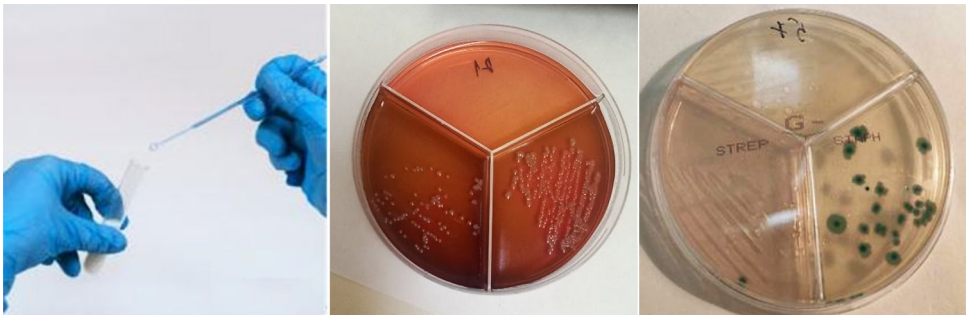

Fig. 1. Cultivation of samples on MicroMast Plates and ClearMilk Test