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Development and testing of a set of chromogenic media for rapid diagnosis of bovine mastitis

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ABSTRACT

Introduction. Bovine mastitis remains one of the most prevalent and economically significant diseases in dairy cattle production. Three chromogenic media have been proposed for the diagnosis, each specifically designed for isolation and differentiation of certain mastitis pathogen groups: Medium I is intended for *Enterobacteriaceae* family bacteria, Medium II – for *Staphylococcus* genus microorganisms, Medium III – for *Streptococcus* genus bacteria.

Objective. The objective is to evaluate the sensitivity, specificity, differentiation capacities and inhibitory properties of these chromogenic media, and to test the media using milk samples from mastitic cows.

Materials and methods. For sensitivity testing, the control strains (*Streptococcus agalactiae*, *Staphylococcus aureus* and *Escherichia coli*) at concentrations of 1×10^0, 1×10^1, and 1×10^2 CFU/mL were used. Microbial growth was assessed following 24-hour incubation at 37 °C. Specificity and differentiation capacities were studied using 22 microbial strains, their growth patterns and colony coloration in chromogenic and control media were compared. Inhibitory properties were determined based on presence/absence of culture growth. The media were evaluated using milk samples from mastitic cows and standardized culturing methods.

Results. The chromogenic media demonstrated sensitivity comparable to the control media (Columbia agar supplemented with 5% defibrinated sheep blood), $p > 0.05$. Medium I enabled reliable color-based differentiation but showed limited inhibitory effects. Medium II ensured selective isolation of staphylococci while effectively suppressing growth of other bacteria. Medium III supported growth of both enterococci and streptococci, including *Streptococcus agalactiae*. The tests conducted in milk samples confirmed genus level differentiation capability.

Conclusion. The developed chromogenic media ensure high-accuracy mastitis diagnosis due to their sensitivity, specificity and differentiation properties. Their implementation makes it possible to cover an extensive range of microorganisms and to selectively isolate the targeted bacterial groups. Further work will be aimed at improving the media for fungal growth suppression and increasing the diagnostic accuracy.

Keywords: mastitis, rapid diagnosis, cattle, milk, chromogenic media

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Разработка и апробация набора хромогенных сред для экспресс-диагностики мастита крупного рогатого скота

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РЕЗЮМЕ

Введение. Мастит крупного рогатого скота является одним из наиболее распространенных и экономически значимых заболеваний в молочном животноводстве. Для его диагностики предложены три хромогенные среды, каждая из которых предназначена для выделения и дифференциации определенных групп возбудителей мастита: среда I – для бактерий семейства *Enterobacteriaceae*, среда II – для микроорганизмов рода *Staphylococcus*, среда III – для бактерий рода *Streptococcus*.

Цель исследования. Оценка чувствительности, специфичности, дифференцирующих и ингибирующих свойств хромогенных сред, а также их апробация на образцах молока от коров с маститом.

Материалы и методы. Для оценки чувствительности использовали контрольные штаммы *Streptococcus agalactiae*, *Staphylococcus aureus* и *Escherichia coli* в различных концентрациях (1×10^0, 1×10^1, 1×10^2 КОЕ/мл). Рост микроорганизмов оценивали через 24 ч инкубации при 37 °C. Специфичность и дифференцирующие свойства изучали на 22 штаммах микроорганизмов, сравнивая их рост и цвет колоний на хромогенных и контрольной средах. Ингибирующие свойства оценивали по наличию или отсутствию роста культур. Апробацию сред проводили с использованием образцов молока от коров с маститом, используя стандартизированные методы посева и культивирования.

Результаты. Хромогенные среды показали сопоставимую с контрольной средой (колумбийский агар с добавлением 5% дефибринированной крови барана) чувствительность ($p > 0,05$). Среда I обеспечила дифференциацию микроорганизмов по цвету колоний, но имела низкие ингибирующие свойства. Среда II избирательно выделяла стафилококки, подавляя рост других бактерий. Среда III поддерживала рост энтерококков и стрептококков, в том числе *Streptococcus agalactiae*. Апробация на образцах молока подтвердила возможность дифференциации культур до вида.

Заключение. Разработанные хромогенные среды обеспечивают высокую точность диагностики мастита, сочетая чувствительность, специфичность и дифференцирующие свойства. Их комплексное использование позволяет охватить широкий спектр микроорганизмов и избирательно выделить целевые группы бактерий. Дальнейшая работа будет направлена на улучшение сред для подавления роста грибов и повышения точности диагностики.

Ключевые слова: мастит, экспресс-диагностика, крупный рогатый скот, молоко, хромогенные среды

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INTRODUCTION

Bovine mastitis, an inflammatory condition of the mammary gland, ranks among the most widespread and economically impactful diseases in dairy production [1, 2, 3]. Transmission occurs due to multiple factors, including poor milking hygiene, suboptimal housing conditions, improper milking techniques, weakened animal immunity and inadequate preventive measures [4, 5, 6]. The disease presents in both clinical form – characterized by visible symptoms such as udder swelling, redness, and pain – and subclinical form, which lacks overt inflammation but results in reduced milk quality [7].

The etiology of mastitis comprises two primary causative groups: mechanical and infectious. Mechanical causes involve udder injuries resulting from inappropriate milking techniques, defective milking equipment or traumas during grazing. These injuries establish favorable conditions for microbial invasion, potentially leading to inflammatory development [8]. Nevertheless, pathogenic microorganisms constitute the principal factor in mastitis occurrence [9].

The most common pathogens of mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli* and others [10, 11]. *S. aureus* is one of the most dangerous pathogens, as it can induce chronic forms of mastitis that are resistant to treatment [12, 13, 14, 15]. *S. agalactiae* is transmitted mainly through milking equipment and can persist in cows for a long time [16]. *E. coli* often causes acute forms of mastitis, accompanied by severe symptoms [17].

To diagnose infectious bovine mastitis, veterinarians have multiple diagnostic tools at their disposal, each tool having its distinct advantages and limitations [18, 19, 20, 21, 22, 23, 24]. Among these, bacteriological milk testing remains one of the most widely used methods [25, 26]. This procedure involves aseptic milk sample collection followed by inoculation onto culture media. After thermostat incubation, microorganisms are identified based on their morphological, biochemical and cultural properties. While this method enables precise pathogen identification and facilitates targeted treatment selection, it requires specialized equipment and has a relatively long turnaround time (2–3 days) [27]. Chromogenic media can be used to speed up the diagnosis of infectious mastitis. These specialized media contain substrates that undergo color changes when acted upon by pathogen-specific enzymes, allowing for etiological agent identification within 24 hours post-inoculation. Current rapid test options include Compact Dry (R-Biopharm AG, Germany) and RIDA® COUNT (Chisso Corporation, Japan) test plates [28, 29]. These test systems feature various specialized assays for determining *S. aureus*, *Enterobacteriaceae*, *Salmonell*a, total microbial count, *E. coli*, as well as yeast and mold contamination.

The Laboratory for Diagnostics and Control of Antibiotic Resistance of Pathogens of the Most Clinically Significant Infectious Animal Diseases, Federal Scientific Centre VIEV has developed its own formulation of chromogenic media for differentiating mastitis pathogens without requiring lengthy laboratory studies. The set consists of three distinct chromogenic media that, when used together, enable identification of the mastitis pathogen spectrum in each specific case. This approach facilitates determination of the pathogenic spectrum, thereby influencing subsequent therapeutic decisions.

The study aims to evaluate the efficacy and diagnostic quality of these chromogenic media for bovine mastitis diagnosis.

MATERIALS AND METHODS

Chromogenic media. Three chromogenic nutrient media were prepared.

Medium I is intended for the determination and differentiation of the most frequently encountered microorganisms of the *Enterobacteriaceae* family.

Medium II is intended for the determination and differentiation of microorganisms of the genus *Staphylococcus*.

Medium III is intended for the determination and differentiation of microorganisms of the genus *Streptococcus* (in particular, *S. agalactiae*).

For ease of use, the three media were placed in one Petri dish with sectors.

The efficacy of chromogenic nutrient media was determined according to the following criteria: sensitivity, specificity, cultural properties of microorganism control strains, differentiating and inhibitory properties. A commercially available medium, Columbia blood agar (HiMedia Laboratories Pvt Ltd., India) supplemented with 5% defibrinated ram blood served as control.

Control strains. The control strains comprised 22 microbial cultures from the collection of pathogenic and vaccine strains maintained at the Federal Scientific Center VIEV, including: *E. coli* ATCC 25922, *S. agalactiae* ATCC 8057, *S. aureus* ATCC 12600, *Klebsiella pneumoniae* B-1392, *Proteus mirabilis* B-1382, *Pseudomonas aeruginosa* B-1366, *Salmonella typhimurium* B-1025, *Enterococcus faecalis* B-1399, *Enterococcus faecium* 1921, *Acinetobacter baumannii* 2516, *Enterobacter cloacae* 1322, *Staphylococcus hominis* 1377, *Staphylococcus equorum* 2511, *Staphylococcus haemolyticus* 2505, *Staphylococcus pseudintermedius* B-1849, *Morganella morganii* 1418, *Streptococcus uberis* 2114, *Streptococcus dysgalactiae* 2432, *Streptococcus pyogenes* 1972, *Aerococcus viridans* 2320, *Streptococcus canis* 2326, *Streptococcus suis* 2383.

Preparation of bacterial suspension dilutions. Initial bacterial suspensions were prepared at concentrations of 1×10^8 to 1×10^9 CFU/mL using the pharmacopoeial reference standard (PhRS 3.1.00085). To achieve the required seeding densities, serial ten-fold dilutions of the initial suspensions were performed.

Determination of sensitivity. S. agalactiae, *S. aureus* and *E. coli* strains were inoculated onto the studied chromogenic and control media in 1 mL at different concentrations: 1×10^0, 1×10^1, 1×10^2 CFU/mL. After (24 ± 2) hours of incubation at 37 °C, the number of grown colonies in all the inoculations was compared. The experiment was performed in triplicate. To compare the mean values of the groups and determine statistically significant differences between them, the Student's test (*t*-criterion) was used: the differences are considered statistically significant if p-value < 0.05.

Specificity assessment. Specificity was determined for each chromogenic medium separately. The growth and nature of changes in colonies of different bacterial strains on the same experimental medium were compared and the presence of similarities or differences was noted.

Evaluation of differentiating properties. To determine the differentiating properties, changes in control strains inoculated in chromogenic and control media (structure, color of colonies, color of the medium around the colonies) were compared.

Evaluation of inhibitory properties. Inhibitory properties were determined by the presence or absence of growth of cultures on chromogenic media in comparison with the presence of growth on a control medium.

Testing of media with mastitis milk samples. Eight milk samples (10 mL each) were collected from cows with mastitis confirmed by the Kenotest somatic cell test (CID Lines, Belgium). Samples were collected in sterile biological specimen containers and processed within 2 hours of collection, with storage maintained at +4 to +8 °C. For inoculation, a sterile cotton swab was immersed in each milk sample, excess moisture was removed by touching the container walls, and then streaked in a lawn pattern onto three chromogenic media. The cultures were incubated at 37 °C for 24 hours before result interpretation.

RESULTS AND DISCUSSION

To determine the sensitivity of chromogenic media, three target strains (*S. agalactiae*, *S. aureus*, and *E. coli*) inoculated in 1 mL at three different concentrations (1×10^0, 1×10^1, 1×10^2 CFU/mL) were used. After culturing for 24 hours at 37 °C, the number of colony-forming units for all media was counted. The results are presented in Table 1.

To identify statistically significant differences or similarities, the Student's *t*-test was used, the results of which are presented in Table 2.

Table 1
Mean colony-forming unit values for each tested microorganism species in experimental and control media

CFU/mL		Medium I	Medium II	Medium III	Control
S. agalactiae	1×10^2	116.0 ± 11.4	Inhibited	117.7 ± 12.7	105.3 ± 21.4
	1×10^1	20.3 ± 1.2		21.7 ± 3.8	23.0 ± 6.1
	1×10^0	3.7 ± 1.2		5.7 ± 1.5	3.3 ± 3.5
S. aureus	1×10^2	112.0 ± 14.0	111.3 ± 8.3	Inhibited	118.3 ± 10.0
	1×10^1	21.7 ± 3.2	17.3 ± 2.1		23.0 ± 6.1
	1×10^0	3.7 ± 2.5	4.0 ± 2.6		3.0 ± 2.6
E. coli	1×10^2	100.3 ± 4.9	Inhibited	Inhibited	118.3 ± 9.1
	1×10^1	27.0 ± 2.0			21.7 ± 1.5
	1×10^0	4.3 ± 2.1			2.3 ± 2.3

Table 2
Statistical significance assessment (Student's *t*-test) between compared groups

Compared groups	CFU/mL	*S. agalactiae*	*S. aureus*	*E. coli*
Medium I *vs* control	1×10^2	0.63	0.18	0.06
	1×10^1	0.45	0.81	0.12
	1×10^0	0.89	0.42	0.18
Medium II *vs* control	1×10^2	Inhibited	0.46	Inhibited
	1×10^1		0.30	
	1×10^0		0.76	
Medium III *vs* control	1×10^2	0.39	Inhibited	Inhibited
	1×10^1	0.84		
	1×10^0	0.48		

Based on the obtained data, chromogenic media demonstrate sensitivity comparable to the control medium, as confirmed by statistical analysis (Student's *t*-test, $p > 0.05$). The observed differences between chromogenic media and the control medium showed no statistical significance across all tested strains and concentrations. Thus, these results indicate that chromogenic media effectively support growth of target microorganisms even at minimal inoculum levels.

The specificity, differentiating properties and inhibitory characteristics of the chromogenic media were evaluated concurrently using 22 microbial strains representing diverse species. The results are presented in Table 3.

Medium I was found to be highly specific: most of the tested bacteria formed colonies with unique colors. For example, *E. coli* formed burgundy colonies, *S. aureus* – golden, *P. aeruginosa* – gray-green, and *S. equorum* – violet-pink. However, some microorganisms, such as *E. cloacae* and *K. pneumoniae*, had similar colony colors (violet-blue), which may make it difficult to distinguish them visually. Inhibitory properties were weak: all studied strains

Table 3
Results of tests for specificity, differentiation capacities and inhibitory properties of chromogenic media as compared with control medium

Microorganisms	Medium I		Medium II		Medium III		Control	
	Growth	Colony color	Growth	Colony color	Growth	Colony color	Growth	Colony color
Escherichia coli	Good	Burgundy	Inhibited		Inhibited		Good	Grayish-white
Klebsiella pneumoniae	Good	Violet-blue	Inhibited		Inhibited		Good	Grayish-white
Proteus mirabilis	Good	Transparent	Good	Transparent	Moderate	Transparent	Good	Grayish-white
Pseudomonas aeruginosa	Good	Grey-green	Inhibited		Good	Blue-green	Good	Blue-green
Salmonella typhimurium	Good	Transparent	Inhibited		Inhibited		Good	Grayish-white
Enterococcus faecalis	Good	Blue-light blue	Inhibited		Good	Blue-green	Good	Grayish-white
Enterococcus faecium	Good	Blue-green	Inhibited		Good	Blue-green	Good	Grayish-white
Acinetobacter baumannii	Good	Pale-yellow	Inhibited		Inhibited		Good	Grayish-white
Enterobacter cloacae	Good	Violet-blue	Inhibited		Inhibited		Good	Grayish-white
Morganella morganii	Good	Amber	Inhibited		Moderate	White	Good	Grayish-white
Staphylococcus aureus	Good	Golden	Moderate	Violet	Inhibited		Good	Golden
Staphylococcus hominis	Good	White	Good	Blue-green	Inhibited		Good	White
Staphylococcus equorum	Good	Violet-pink	Good	Blue-green	Inhibited		Good	White
Staphylococcus haemolyticus	Good	White	Good	Green	Inhibited		Good	Grayish-white
Staphylococcus pseudintermedius	Good	Beige-pink	Good	Blue-green	Inhibited		Good	Grayish-white
Streptococcus agalactiae	Moderate	Pale-pink	Inhibited		Good	Blue	Good	Grayish-white
Streptococcus uberis	Moderate	White	Inhibited		Moderate	White	Good	Grayish-white
Streptococcus dysgalactiae	Moderate	Pale-pink	Inhibited		Moderate	White	Good	Grayish-white
Streptococcus pyogenes	Moderate	White	Inhibited		Moderate	White	Good	Grayish-white
Aerococcus viridans	Moderate	White	Inhibited		Inhibited		Good	Greenish
Streptococcus canis	Moderate	White	Inhibited		Moderate	White	Good	Grayish-white
Streptococcus suis	Moderate	Pale-pink	Inhibited		Moderate	White	Good	Grayish-white

of microorganisms demonstrated growth within 24 hours. Nevertheless, medium I ensured effective differentiation of control strains by colony color, which allows visually distinguishing microorganisms already at early stages.

The inhibitory properties of Medium II are pronounced: the growth of most bacteria was absent, with the exception of the target microorganisms – *Staphylococcus* spp. It is worth noting that the specificity of the medium is low – most staphylococci were stained blue-green. However, the same color was predominantly saprophytic microorganisms, while potentially pathogenic staphylococci (*S. aureus* and *S. haemolyticus*) differed in color. For example, *S. aureus* formed purple colonies, and *S. hominis* and *S. equorum* formed blue-green ones, which made it possible to visually distinguish them. Medium II as compared with the control one, provided a differentiation of staphylococci by color.

Medium III demonstrated good inhibitory properties, effectively suppressing the growth of most microorganisms, with the exception of gram-positive cocci and some representatives of the *Enterobacteriaceae* family. The differentiating and specific properties of the medium were weakly expressed and manifested mainly for enterococci, which were stained blue-green, and for *S. agalactiae*, which formed blue colonies.

The use of all three chromogenic media in combination provides a comprehensive approach to mastitis diagnosis, demonstrating high sensitivity, specificity and differentiating properties. This method allows for a wide range of microorganisms to be covered, selectively isolating target bacterial groups such as staphylococci, streptococci and enterococci.

For testing in the field, milk samples were collected and then inoculated onto three chromogenic media. The results are shown in the Figure.

The simultaneous use of three media for milk sample inoculation enables nearly species-level differentiation of cultures. In Figure (a) it is evident that *Enterococcus* sp. grew on Media I and III (supposedly *E. faecalis*, as *E. faecium* typically exhibits a darker green coloration). Single colonies on Medium II consist of *Staphylococcus* sp., while the presence of *S. aureus* can be ruled out, as it would appear purple on Medium II. Additionally, burgundy colonies on Medium I indicate the presence of *E. coli* in the sample.

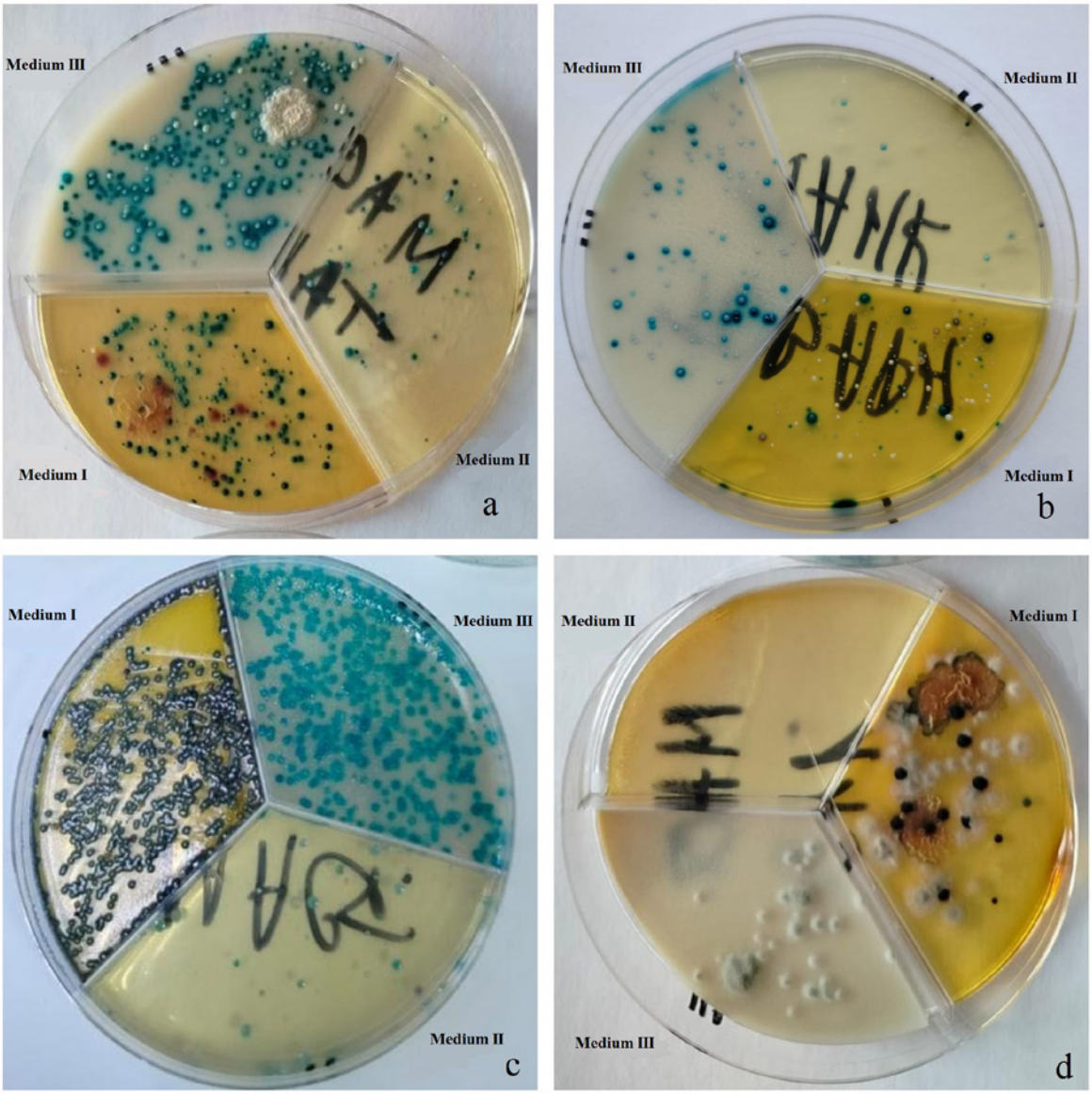

Fig. Testing of culture media using milk samples (cultivated at 37 °C for 24 hours)

In Figure (b), the sample microbiome consists almost exclusively of *Enterococcus* sp. White and green colonies on Media I and II, respectively, are formed by *Staphylococcus* sp. microorganisms, excluding *S. aureus*. Figure (c) reveals a monoculture of *Enterococcus* sp., most likely *E. faecium*. The fourth sample shown in Figure (d), contained only filamentous fungi. Another milk sample yielded results similar to (b), while three other cultures showed no growth.

CONCLUSION

The developed chromogenic media demonstrate high efficiency in bovine mastitis diagnosis. Medium I, with its high sensitivity and differentiating properties, enables primary screening and detection of a broad spectrum of microorganisms, including members of the *Enterobacteriaceae* family. Medium II, due to its strong inhibitory properties, selectively isolates staphylococci, which is a critical feature for identifying pathogenic species such as *S. aureus*. Medium III, while having more limited differentiating capabilities, effectively supports the growth of enterococci and streptococci, including *S. agalactiae*, making it essential for mastitis diagnosis.

The integrated use of all three media ensures high diagnostic accuracy, enabling not only broad microbial coverage but also selective identification of target bacterial groups. This significantly accelerates pathogen detection and facilitates timely administration of effective therapy. Testing of the media on milk samples from mastitic cows confirmed the media's practical applicability and effectiveness when used in the field.

During the testing, occasional development of filamentous fungi was observed, which may complicate result interpretation. To address this, further work will focus on optimizing the media composition by evaluating various antifungal preparations at different concentrations. These improvements aim to enhance media specificity by suppressing non-target fungal growth, thereby reducing the risk of false-positive results.

It is worth noting that standardized disposable loops for milk culture make it possible to roughly estimate the number of colony-forming units. While this method lacks high precision, it provides a practical approximation of milk contamination levels, offering valuable preliminary insights into infection severity.

Thus, the developed chromogenic media represent a promising tool for rapid mastitis diagnosis, combining high sensitivity, specificity and differentiating capabilities. Their implementation in veterinary practice could significantly accelerate diagnostic procedures and enhance mastitis treatment efficacy, ultimately improving animal health and dairy herd productivity.

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