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Evaluation of chromogenic culture media for rapid identification of microorganisms isolated from cows with clinical and subclinical mastitis

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ABSTRACT

This study aimed to evaluate the diagnostic performance (specificity, Sp; sensitivity, Se; accuracy; positive predictive value; negative predictive value; and Cohen's kappa coefficient, κ, of agreement) of chromogenic culture media for rapid identification of microorganisms isolated from cows with clinical (CM) and subclinical mastitis (SCM). For this, 2 experiments were carried out: evaluation of (1) biplate, and (2) triplate of chromogenic culture media for rapid identification of mastitis-causing microorganisms. For the evaluation of diagnostic performance, identification of microorganisms by MALDI-TOF mass spectrometry was considered the standard methodology. In experiment 1, 476 milk samples collected from cows with CM and 660 from cows with SCM were evaluated by inoculation in 2 selective chromogenic culture media (CHROMagar) for gram-positive bacteria and another for gram-negative bacteria. In experiment 2, 476 milk samples from cows with CM and 500 from cows with SCM were evaluated by inoculation in triplate chromogenic culture media (Smartcolor2, Onfarm), selective for *Streptococcus* and Strep-like organisms, *Staphylococcus*, and gram-negative bacteria. In experiment 1 for the CM samples, the use of biplates with gram-positive and gram-negative culture media showed Se that ranged from 0.56 (0.32–0.81; *Staphylococcus aureus*) to 0.90 (0.80–0.99 *Streptococcus uberis*), Sp varied from 0.94 (0.92–0.96; *Strep. uberis*) to 1.00 (*Prototheca* spp. or yeast), and κ ranged from 0.47 (0.26–0.67; *Staph. aureus*) to 0.84 (0.78–0.9; *Escherichia coli*). The Se of biplates for SCM samples ranged from 0.50 (0.15–0.85; *E. coli*) to 0.94 (0.87–1.00; *Staph. aureus*), Sp varied from 0.95 (0.93–0.97; *Strep. uberis*) to 0.99 (0.98–1.00; *Staph. aureus* and *Strep. Agalactiae* or *dysgalactiae*), and κ ranged from 0.18 (0.00–0.40; *Escherichia coli*) to 0.88 (0.80–0.95; *Staph. aureus*). In experiment 2, the Se of the triplate chromogenic media in CM samples ranged from 0.09 (0.00–0.26; *Serratia* spp.) to 0.94 (0.85–1.00; *Klebsiella* spp. and *Enterobacter* spp.), Sp varied from 0.94 (0.92–0.96; *Strep. agalactiae* and *Strep. dysgalactiae*) to 1.00 (*Serratia* spp.) and κ ranged from 0.07 (0.00–0.24; *Serratia* spp.) to 0.85 (0.75–0.94; *Klebsiella* spp. and *Enterobacter* spp.). For SCM samples, the use of the triplate with the chromogenic culture media showed Se that varied from 0.25 (0.10–0.40; *Lactococcus* spp.) to 1.00 (*Strep. Agalactiae* or *dysgalactiae*), Sp ranged from 0.92 (0.90–0.94; *Strep. Agalactiae* and *Strep. dysgalactiae*) to 0.99 (0.98–1.00; *Klebsiella* spp. and *Enterobacter* spp.), and κ varied from 0.28 (0.00–0.72; *E. coli*) to 0.72 (0.60–0.82; *Staph. aureus*). Our results suggest that the diagnostic accuracy of the biplate and triplate of chromogenic culture media varies according to pathogen, and the results of chromogenic culture media may be useful for rapid decision-making on mastitis treatment protocols of the main mastitis-causing microorganisms, but their use for implementation of mastitis control measures will depend on each farm specific needs.

Key words: on-farm culture, mastitis, chromogenic media, dairy cow

INTRODUCTION

Bovine mastitis is one of the most prevalent and damaging diseases in dairy herds. The main costs associated with mastitis are related to production losses and reduced milk quality and costs for discarded milk containing antimicrobial residues, medicines, and veterinary services, in addition to costs associated with cows with severe mastitis, which can put them at high risk of death (Down et al., 2017).

Mastitis is one of the main reasons for the use of antimicrobials on dairy farms. According to the country considered, the main use of antimicrobials in dairy cows is for the treatment of clinical mastitis (**CM**) during lactation (Saini et al., 2012; Tomazi and dos Santos, 2020) or for dry cow therapy (Kuipers et al., 2016).

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However, not all cases of clinical mastitis require treatment with antimicrobials (Jamali et al., 2018). A recent study on the incidence of CM in Brazil reported that 44% of CM cases had no bacterial growth and about 7% of cases with positive microbiological culture were caused by other microorganisms of nonbacterial origin. Thus, the rapid diagnosis of mastitis-causing microorganisms allows the rational use of antimicrobials and the adoption of appropriate measures for treatment (Tomazi et al., 2018).

Currently, most dairy farms do not routinely perform the diagnosis of the microorganisms that cause CM, which implies the use of antimicrobial treatment protocols without prior knowledge of the mastitis-causing agent (McCarron et al., 2009). On the other hand, collection and shipping milk samples to specialized microbiology laboratories also pose limitations, considering the long time interval between sending the sample and receiving results (Lago et al., 2011)

The use of on-farm microbiological culture systems allows the presumptive identification of the main groups of mastitis-causing microorganisms within 24 h. These systems include the use of selective culture media to differentiate microorganisms into categories and, in some cases, allow identification at the species level (Royster et al., 2014). Thus, according to the types of causative microorganisms, it is possible to define which cases need treatment with antimicrobials, allowing the use of more appropriate treatment protocols and reducing unnecessary use of antimicrobials, which occurs when the causative agent is not isolated or when the use of antimicrobials is not recommended (Lago et al., 2011). In addition to improved treatment protocols for clinical mastitis, rapid culture identification of specific mastitis-causing pathogens may be useful to identify cows to cull or segregate from the herd.

Chromogenic culture media were developed to identify pathogens according to the specific color of the microbial colonies. The chromogenic substrate, when it contacts a specific microorganism after undergoing hydrolysis, releases a dye that sets in the microbial colonies, differentiating them by color (Perry and Freydière, 2007). Compared with conventional culture media, chromogenic media offer rapid diagnosis, reducing the use of biochemical and serological tests to identify pathogens (Perry, 2017). However, studies on the use of chromogenic media to identify pathogens that cause CM and SCM are still scarce and recent.

The hypothesis of this study is that chromogenic media have adequate diagnostic accuracy for the identification of the main mastitis-causing agents compared with the reference standard methodology, which would make it useful for on-farm diagnosis. Thus, this study aimed to evaluate diagnostic accuracy, by determining the accuracy (**Ac**), sensitivity (**Se**), specificity (**Sp**), positive predictive value (**PPV**), negative predictive value (**NPV**), and level of agreement (Cohen's kappa coefficient, κ) of the identification of bovine mastitis-causing microorganisms using biplates and triplates of chromogenic culture media.

MATERIALS AND METHODS

The experimental protocol of the present study was approved by the Ethics Committee on the Use of Animals of the Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo. The study was organized in 2 experiments:

(1) Evaluation of biplate petri dish containing chromogenic culture media (Mastitis GP and Mastitis GN, both from CHROMagar) for rapid identification of mastitis-causing microorganisms. In the period from June 2018 to February 2019, 1,181 milk samples were evaluated. Of these, 476 were from cows with CM and 660 from cows with SCM. All milk samples were submitted to identification of mastitis-causing microorganisms, based on the results of the biplate with chromogenic culture media [gram-positive (**GP**) and gram-negative (**GN**)] and standard methodology (MALDI-TOF MS).
(2) Evaluation of triplate petri dish containing chromogenic culture media (Smartcolor2, Onfarm), for rapid identification of mastitis-causing microorganisms. From June to October 2019, 976 milk samples from cows with mastitis were analyzed, of which 476 samples were from cows with CM and 500 from cows with SCM. All samples were analyzed by standard methodology and by the triple plate with chromogenic culture media triplate.

Sample Selection

Milk samples used in experiments 1 and 2 were selected as a convenience sample (based on farm proximity to the laboratory), which included samples collected from cows with CM (25 farms) and SCM (15 farms) sent for microbiological culture to the Milk Quality Research Laboratory (Qualileite), University of São Paulo, Pirassununga, Brazil. The milk samples were collected by farm personnel and sent to the laboratory for microbiological culture. The samples were frozen at −20°C for up to 30 d until they were inoculated in the culture media. The recommended sample collection protocol was carried out aseptically. The teats were immersed in teat disinfectant solution for 30 s and then dried with paper towels. Then, the 3 initial milk

streams were discarded. The teat ends were disinfected with 70% iodized alcohol, and the milk was collected in a sterile tube (NMC, 2017).

The cases of CM and SCM were identified according to the criteria used by each farm, independently. Clinical mastitis was identified when cows presented milk with abnormal characteristics, such as the presence of clots, blood, or abnormal color secretion, with or without inflammation, pain in the affected mammary quarter, or both. Subclinical mastitis milk samples were defined when cows had milk SCC $>200 \times 10^3$ cells/mL, without the presence of visual changes in the characteristics of milk.

Experiment 1: Evaluation of Biplate Chromogenic Culture Media

The sample collection period and microbiological analysis lasted 8 mo (from June 2018 to February 2019). A total of 476 CM milk samples used in experiment 1 were selected as a convenience sample, from dairy herds located in the states of São Paulo and Minas Gerais. In addition, 660 composite milk samples ($\geq$2 quarters) from cows with SCM, collected from 2 dairy herds, one from the state of São Paulo and the other from Minas Gerais, were included.

Two chromogenic culture media were evaluated. The first medium was selective for gram-positive bacteria (Mastitis GP, CHROMagar) and the second selective for gram-negative bacteria, yeasts, and *Prototheca* spp. (Mastitis GN, CHROMagar). The selective chromogenic culture media GP and GN were plated in 90 × 15 mm biplate petri dishes. The results of microbiological identification were based on the visual assessment of the colony-staining characteristics, according to the indication of the manufacturer.

The interpretation of GP chromogenic culture results was carried out based on the following colony colors as described by the manufacturer's recommendations: (a) dark blue or metallic = *Streptococcus uberis* or *Enterococcus* spp.; (b) turquoise blue = *Streptococcus agalactiae* or *Streptococcus dysgalactiae*; (c) pink = *Staphylococcus aureus*. The GN chromogenic culture identification was made based on the following colony colors: (a) purple = *Escherichia coli*; (b) dark blue or metallic = *Klebsiella* spp., *Enterobacter* spp., or *Serratia* spp.; (c) yellow, cream, or translucent = *Pseudomonas* spp.; (d) white and opaque = yeast or *Prototheca* spp. (Figure 1). The species described above are the only ones that the biplate is intended to identify. In both chromogenic media, colonies with other colors not described above were classified as other GP or GN organisms.

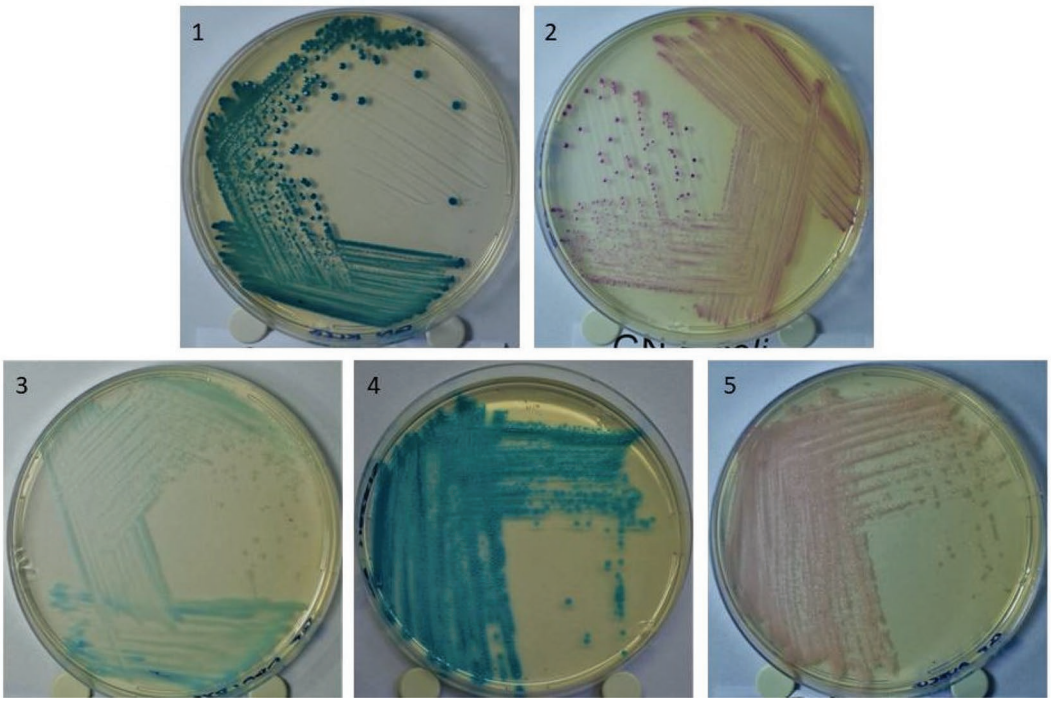

Figure 1. Visual assessment of the characteristic colors of colonies of mastitis-causing microorganisms inoculated in biplate of chromogenic culture media: (1) *Klebsiella* spp., *Enterobacter* spp., or *Serratia* spp.; (2) *Escherichia coli*; (3) *Streptococcus agalactiae* or *Streptococcus dysgalactiae*; (4) *Streptococcus uberis*; and (5) *Staphylococcus aureus*.

The selected milk samples were inoculated in the culture media using a sterile swab. After inoculation, the biplates were incubated at 37°C for 24 h under aerobic conditions. After the incubation period, the growth of colonies was visually inspected using a white background to aid in counting and differentiating colony colors. All colony colors of biplate chromogenic culture media were evaluated according to the manufacturer's recommendations, by a single researcher without knowledge of the microbial identification by MALDI-TOF MS, and the results of bacterial growth were recorded using a digital camera.

Experiment 2: Evaluation of Triplate Chromogenic Culture Media

The period of sample collection and microbiological analysis lasted 4 mo (from July to November 2019). A total of 476 CM milk samples used in experiment 2 were selected as a convenience sample from 12 dairy herds located in the states of Paraná, São Paulo, and Minas Gerais, as well as 500 milk samples from cows with SCM, distributed in composite samples (>1 quarter; n = 449) and individual quarters (n = 51).

The triplate evaluated (Smartcolor2, Onfarm) was composed of selective media for (a) *Streptococcus* spp., (b) *Staphylococcus aureus* and *Staphylococcus* spp., and (c) gram-negative bacteria.

The results of microbiological identification of the culture media selective for *Staphylococcus* were obtained based on the following colonies colors as described by the manufacturer's recommendations: (a) pink = *Staph. aureus*; (b) other colors = *Staphylococcus* spp. The results of selective culture media for *Streptococcus* were evaluated based on the following colony colors: (a) dark blue = *Strep. uberis*; (b) turquoise blue = *Strep. Agalactiae* or *Strep. dysgalactiae*; (c) purple = *Enterococcus* spp.; (d) lilac = *Lactococcus* spp. Finally, the results of selective culture media for GN were evaluated based on the following colony colors: (a) purple = *E. coli*; (b) metallic blue = *Klebsiella* spp., *Enterobacter* spp., or *Serratia* spp.; (c) yellow = *Pseudomonas* spp.; (d) white and dry = yeast and *Prototheca* spp. (Figure 2). The species above described are the only ones that

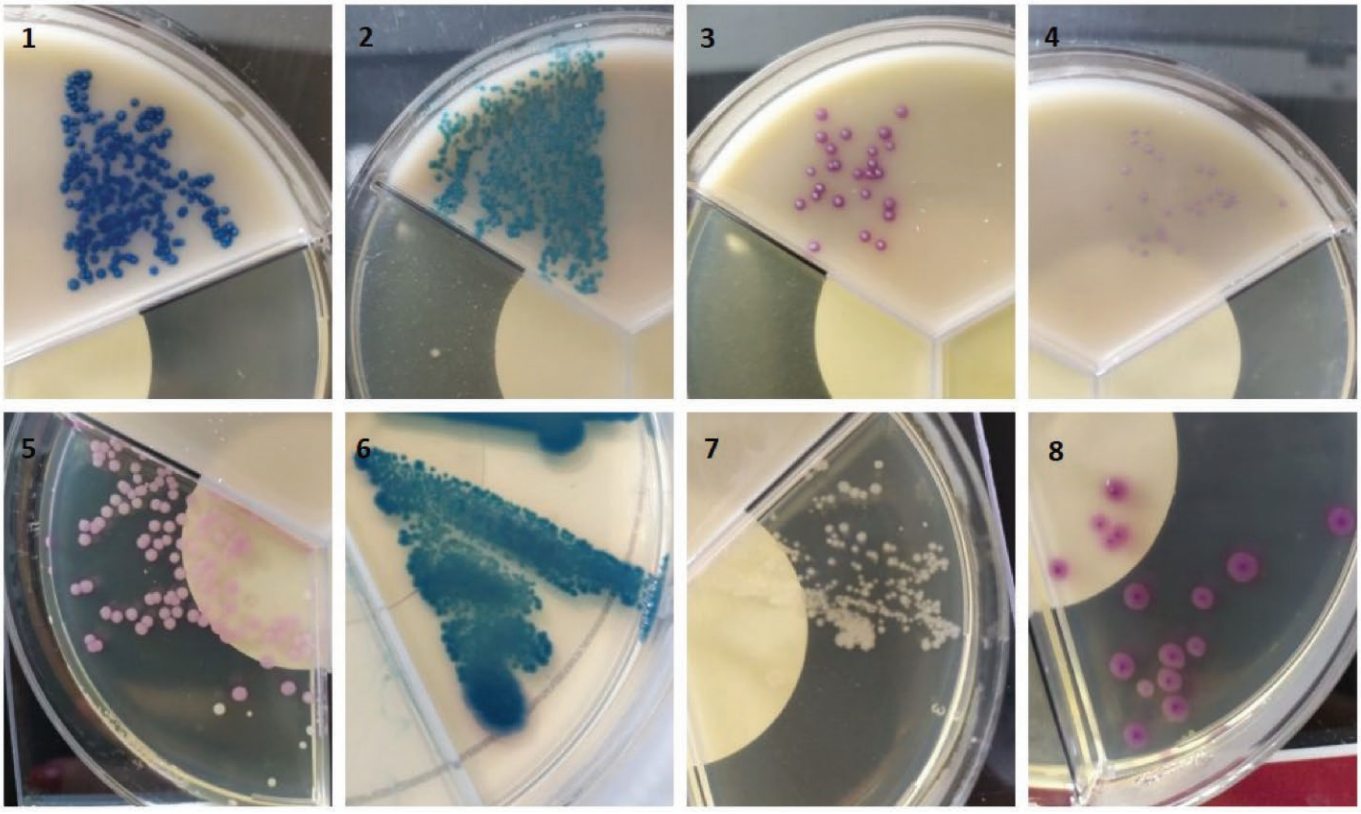

Figure 2. Visual assessment of the characteristic colors of colonies of mastitis-causing microorganisms inoculated in triplate of chromogenic culture media: (1) *Streptococcus uberis*, (2) *Streptococcus agalactiae* or *Streptococcus dysgalactiae*, (3) *Enterococcus* spp., (4) *Lactococcus* spp., (5) *Staphylococcus aureus*, (6) *Klebsiella* spp. or *Enterobacter* spp., (7) *Prototheca* spp. or yeast, and (8) *Escherichia coli*.

the triplate is intended to identify. Any colonies classified with other colors not previously described were classified as other GP or GN microorganisms.

The selected milk samples were inoculated into the culture media with the aid of sterile swabs. Two milk samples were inoculated per triplate and incubated at 37°C for 24 h.

After the incubation period, the growth of colonies of the microorganism were visually inspected with the aid of a white background to differentiate the color and count the microbial colonies. All colony color evaluations for triplate chromogenic culture media were similar to the biplate evaluation procedures.

All milk samples were inoculated at the same time in the chromogenic media and in blood agar (**BA**; blood agar base, Kasvi) enriched with 5% bovine blood, using a sterile swab. After inoculation, the plates were incubated at 37°C for 24 h. All samples with positive microbial growth in BA had microbial colonies identified using MALDI-TOF MS. Quarter or composite milk samples that presented growth of ≥3 colonies with distinct morphological characteristics in any culture media (chromogenic media or BA) were considered contaminated and discarded.

Microbial Identification by MALDI-TOF MS

After 24-h incubation, microbial isolates that showed positive growth in BA were selected for microbiological identification by MS, as described by Barcelos et al. (2019). For the ribosomal protein extraction, a colony was selected from each isolate and applied to a steel plate containing 96 wells (MSP 96 Target polished steel, Bruker Daltonik) with a sterile wooden stick. Colonies were selected according to their color; when 2 different colonies were observed, 1 colony of each type was selected and evaluated separately. After transferring the colony to the steel plate, a volume of 1.0 μL of formic acid (70%) was added to the spot and subjected to dry at room temperature. After drying, 1.0 μL of α-cyano-4-hydroxycinnamic acid matrix (**HCCA**; diluted in 50% acetonitrile and 2.5% trifluoroacetic acid) was added and left to dry again at room temperature. An *E. coli* isolate was used as a positive control, and a spot containing only HCCA matrix was used as a negative control. For the MALDI-TOF MS calibration, 1.0 μL of standard protein solution (bacterial test standard, BTS; Bruker) was used, followed by the addition of 1.0 μL of HCCA matrix.

The samples were analyzed on the MicroFlex LT equipment (Bruker Daltonik), and the mass spectra were captured by the FlexControl 3.4 software (Bruker Daltonik) with a target in the range of 2,000 to 20,000 m/z (mass to charge ratio). The microorganisms were identified using the MALDI Biotyper Software 4.1.7 (Bruker Daltonik). The identification results obtained were expressed by scores, such that a score ≥1.7 was considered reliable for genus identification and a score ≥2.0 was reliable for genus and species identification (Barcelos et al., 2019). When the microorganism was not identified at the first evaluation by MALDI-TOF MS, the identification protocol was repeated. If a score between 1.7 and 2.0 was observed in the new identification, the suggestive species with the highest score was considered.

For samples with colony growth in the BA but no identification by MALDI-TOF MS, unidentified colonies were submitted to conventional microbiological identification, based on colony morphology and hemolytic patterns on blood agar, and Gram staining (NMC, 2017). *Prototheca* spp. identification was suspected when colonies in BA were opaque gray and 0.5 to 1 mm in diameter. Suspected colonies were inoculated in Sabouraud agar, and *Prototheca* spp. were identified based on the observation of yeast-like colonies, with creamy consistency, white coloration, and characteristic oval cells under microscopy.

Diagnostic Performance of Chromogenic Media

The indicators of diagnostic performance (Ac, Se, Sp, PPV, and NPV) for identification of mastitis-causing microorganisms in the chromogenic media in both experiments were estimated considering microbiological identification by MALDI-TOF MS as the standard methodology.

The values of Ac, Se, Sp, PPV, and NPV were calculated based on the results of identification in the chromogenic media and standard methodology, according to true positive (**TP**), when there was agreement of results of the same microorganism identified in the chromogenic culture media and standard methodology; true negative, when there was no growth of microorganisms in the chromogenic culture media and in the standard methodology; false positive, when there was a growth of any microorganisms in the standard methodology other than that diagnosed in chromogenic media; false negative (**FN**), when there was no microorganism growth in the chromogenic media and there was growth in the standard methodology (Ferreira et al., 2018). The results of the diagnostic performance (Ac, Se, Sp, PPV, and NPV) were classified as low (<0.60), intermediate (>0.60), or high (>0.80; Royster et al., 2014).

The senspec option of PROC FREQ of SAS version 9.4 (SAS Institute, 2009) was used to calculate Se, Sp, PPV, and NPV. Accuracy was calculated using PROC FREQ, considering the sum of TP and true negative divided by the total number of samples. Confidence

intervals were obtained with their standard errors and Wald confidence, which produces 0.95 confidence limits.

Cohen's kappa coefficient of agreement was calculated using the PROC FREQ of SAS Institute (2009). A value of 1.00 indicates 100% agreement; values between 0.81 and 1.00 are considered to indicate almost perfect agreement, from 0.61 to 0.80 substantial agreement, from 0.41 to 0.60 moderate agreement, from 0.21 to 0.40 fair agreement, from 0.00 to 0.20 slight agreement, and values ≤0.00 poor agreement (Landis and Koch, 1977).

RESULTS

Experiment 1: Evaluation of Biplate Chromogenic Culture Media

A total of 1,136 samples were evaluated; 476 were from CM and 660 were from SCM. In CM samples, 54% (255/476) had growth of a single type of microorganism, 3% (13/476) had growth of 2 microorganisms with distinct morphologies, 4% (19/476) were contaminated, and 40% (189/476) of the samples had no microbial growth. In the SCM samples, we observed microbial growth of colonies with a single morphology in 31% (205/660) of samples, 4% (30/660) with 2 distinct morphologies, 10% (68/660) were contaminated, and 5.4% (30/660) had no microbial growth. The most isolated gram-positive bacteria in CM samples were *Strep. uberis* 6.5% (31/476) and *Strep. dysgalactiae* 6.5% (31/476). On the other hand, in SCM, *Staph. chromogenes* 13.5% (89/660) and *Staph. aureus* 6.5% (43/660) were the most frequently isolated. Among the gram-negative bacteria, the most isolated in CM samples were *E. coli* (0.06) and *Kleb. pneumoniae* (0.05) and, in SCM, other gram-negatives (0.02) and *E. coli* (0.01; Table 1).

Performance of Chromogenic Culture Media Biplates

The Ac of the chromogenic culture media in CM samples ranged from 0.94 (*Strep. Agalactiae* or *dysgalactiae* and *Strep. uberis* and *Enterococcus* spp.) to 0.99 (*Pseudomonas* spp.). The Se of the chromogenic culture media ranged from 0.56 (*Staph. aureus*) to 0.90 (*Strep. Uberis* and *Enterococcus* spp.). The Sp ranged from 0.94 (*Strep. Uberis* and *Enterococcus* spp.) to 1.00 (*Prototheca* spp. or yeast).

The PPV values ranged from 0.59 (*Strep. uberis* and *Enterococcus* spp.) to 0.84 (*E. coli*). Additionally, the results for NPV ranged from 0.96 (*Strep. agalactiae* or *dysgalactiae*) to 0.99 (*Strep. Uberis* and *Enterococcus* spp. and *Prototheca* spp. or yeast; Table 2).

The agreement of results between the standard methodology and the chromogenic culture media (GP and GN) ranged, in the CM samples, from 0.47 to 0.84. Agreement was almost perfect for *E. coli* ($\kappa = 0.84$) and yeast or *Prototheca* spp. ($\kappa = 0.80$), substantial for *Strep. uberis* ($\kappa = 0.67$) and *Strep. agalactiae* and *Strep. dysgalactiae* ($\kappa = 0.67$), and moderate for *Staph. aureus* ($\kappa = 0.47$) and *Pseudomonas* spp. ($\kappa = 0.44$; Table 2).

In the SCM samples, the Ac of the biplate chromogenic culture media ranged from 0.95 (*Strep. uberis* and *Enterococcus* spp.) to 0.99 (*Strep. agalactiae* and *Strep.*

Table 1. Distribution of mastitis-causing agents identified by microbiological milk culture according to the standard MALDI-TOF MS methodology (clinical mastitis, CM = 476; subclinical mastitis samples, SCM = 660), evaluated by biplate chromogenic culture media

Variable	CM	%	SCM	%
Total samples	476	100	660	100
No growth	189	39.7	357	54.1
Colonies with 1 morphology	255	53.6	203	30.8
Colonies with 2 morphologies	13[1]	2.7	32[2]	4.8
Contamination	19	4.0	68	10.3
Gram-positive bacteria	133	27.9	179	27.1
Streptococcus uberis	31	6.5	9	1.4
Streptococcus dysgalactiae	31	6.5	1	0.2
Staphylococcus chromogenes	14	2.9	89	13.5
Staphylococcus aureus	14	2.9	43	6.5
Streptococcus agalactiae	14	2.9	6	0.9
Non-*aureus Staphylococcus*	8	1.7	13	2.0
Other gram-positive bacteria	21[3]	4.4	18[4]	2.7
Gram-negative bacteria	112	23.5	19	2.9
Escherichia coli	70	14.7	4	0.6
Klebsiella pneumoniae	23	4.8	0	0
Serratia marcescens	6	1.3	2	0.3
Enterobacter cloacae	3	0.6	0	0
Klebsiella oxytoca	3	0.6	0	0
Klebsiella varicola	2	0.4	0	0
Other gram-negative bacteria	5[5]	1.1	13[6]	2.0
Other microorganisms	10	2.1	5	0.8
Prototheca spp.	5	1.1	0	0
Candida tropicalis	3	0.6	0	0
Candida krusei	2	0.4	5	0.8

[1]*Escherichia coli* and others (n = 1); *E. coli* and *Staphylococcus aureus* (n = 2); *E. coli* and *Streptococcus dysgalactiae* (n = 2); *Klebsiella* spp. and *Strep. dysgalactiae* (n = 2); NAS and other gram-positive (n = 1); NAS and others gram-negative (n = 1); *Strep. dysgalactiae* and NAS (n = 1); *Strep. dysgalactiae* and others (n = 1); *Strep. uberis* and NAS (n = 1).

[2]*Escherichia coli* and NAS (n = 1); *E. coli* and others (n = 3); *Enterococcus* and others (n = 1); *Lactococcus* and others (n = 1); NAS and NAS (n = 5); NAS and others (n = 3); others and others (n = 7); *Staph. aureus* and *Strep. uberis* (n = 4); *Strep. agalactiae* and *Strep. dysgalactiae* (n = 1); *Strep. dysgalactiae* and NAS (n = 1); *Strep. uberis* and NAS (n = 5).

[3]Mainly gram-positive genera: *Bacillus* spp. (n = 3); *Corynebacterium* spp. (n = 4); *Enterococcus* spp. (n = 4); *Lactobacillus* spp. (n = 1), *Lactococcus* spp. (n = 3); *Macrococcus* spp. (n = 3); *Micrococcus* spp. (n = 1).

[4]Mainly gram-positive genera: *Aerococcus* spp. (n = 5); *Bacillus* spp. (n = 5); *Corynebacterium* spp. (n = 7); *Enterococcus* spp. (n = 1); *Lactococcus* spp. (n = 1); *Macrococcus* spp. (n = 1); *Micrococcus* spp (n = 7).

[5]Mainly gram-negative genera: *Acinetobacter* spp. (n = 2); *Pseudomonas* spp. (n = 2).

[6]Mainly gram-negative genera: *Acinetobacter* spp. (n = 10); *Citrobacter* (n = 1).

Table 2. Diagnostic accuracy (specificity, Sp; sensitivity; Se; accuracy, Ac; positive predictive value, PPV; negative predictive value, NPV; and Cohen's kappa agreement coefficient, κ) of biplate[1] chromogenic media in milk samples from cows with CM (n = 476)

Variable	*Streptococcus agalactiae* or *Streptococcus dysgalactiae*	*Streptococcus uberis* or *Enterococcus* spp.	*Staphylococcus aureus*	*Escherichia coli*	KES[2]	*Prototheca* or yeast
n[3]	49	59	14	80	35	10
n[4]	51	36	16	75	39	11
Ac[5]	0.94 (0.92–0.96)	0.94 (0.92–0.96)	0.97 (0.96–0.99)	0.95 (0.93–0.97)	0.96 (0.95–0.98)	0.99 (0.99–1.00)
Se[5]	0.71 (0.58–0.83)	0.90 (0.80–0.99)	0.56 (0.32–0.81)	0.89 (0.82–0.96)	0.74 (0.60–0.88)	0.73 (0.46–0.99)
Sp[5]	0.97 (0.95 0.98)	0.94 (0.92–0.96)	0.99 (0.98–1.00)	0.97 (0.95–0.98)	0.99 (0.97–1.00)	1.00
PPV[5]	0.73 (0.61–0.86)	0.59 (0.47–0.72)	0.64 (0.39–0.89)	0.84 (0.76–0.92)	0.83 (0.70–0.95)	0.80 (0.55–1.00)
NPV[5]	0.96 (0.94–0.98)	0.99 (0.98–1.00)	0.98 (0.97–1.00)	0.98 (0.96–0.99)	0.98 (0.96–0.99)	0.99 (0.99–1.00)
κ[5]	0.67 (0.56–0.77)	0.67 (0.57–0.78)	0.47 (0.26–0.67)	0.84 (0.78–0.91)	0.74 (0.63–0.85)	0.80 (0.65–0.97)
κ *P*-value[6]	0.724	<0.001	0.038	0.371	0.251	0.655

[1]Biplate with selective chromogenic culture media for gram-positive and gram-negative bacteria (Mastitis GP and Mastitis GN, respectively, both from CHROMagar).

[2]KES = *Klebsiella* spp., *Enterobacter* spp., or *Serratia* spp.

[3]Total number of samples testing positive on the biplate.

[4]Total number of samples testing positive to the gold-standard test (blood agar).

[5]Values in parentheses = 95% CI.

[6]Cohen's Kappa test.

dysgalactiae, and *Klebsiella* spp., *Enterobacter* spp., and *Serratia* spp.). The Se of the chromogenic culture media ranged from 0.20 (*Prototheca* spp. or yeast) to 0.94 (*Staph. aureus*), whereas the Sp ranged from 0.95 (*Strep. uberis* and *Enterococcus* spp.) to 0.99 (*Staph. aureus*).

The PPV values ranged from 0.31 (*E. coli*) to 1.00 (*Prototheca* spp. and yeast). In contrast, the NPV results ranged from 0.99 (*Staph. aureus* and *E. coli*) to 1.00 (*Strep. agalactiae* or *Strep. dysgalactiae* and *Strep. uberis* or *Enterococcus* spp.; Table 3).

In SCM samples, almost perfect agreement in results of methodologies was observed for *Staph. aureus* (κ = 0.88), whereas for *Strep. agalactiae* and *dysgalactiae* (κ = 0.68), substantial agreement was observed. For the remaining microorganisms, we observed slight agreement for *E. coli* (κ = 0.18) and fair agreement for *Prototheca* spp. and yeast (κ = 0.33) and *Strep. uberis* (κ = 0.35). The kappa coefficient was not calculated for *Klebsiella* spp. and *Serratia* spp. because of the small number of isolates, or for *Pseudomonas* spp., because only one true positive result was found (Table 3).

Experiment 2: Evaluation of Triplate Chromogenic Culture Media

From a total of 976 samples, 476 were from CM and 500 were from SCM. Considering CM, there was

Table 3. Diagnostic accuracy (specificity, Sp; sensitivity; Se; accuracy, Ac; positive predictive value, PPV; negative predictive value, NPV; and Cohen's kappa agreement coefficient, κ) of the biplate[1] chromogenic media in milk samples from cows with SCM (n = 660)

Variable	*Streptococcus agalactiae* or *Streptococcus dysgalactiae*	*Streptococcus uberis* or *Enterococcus* spp.	*Staphylococcus aureus*	*Escherichia coli*
n[2]	15	45	52	13
n[3]	9	18	47	8
Ac[4]	0.99 (0.98–1.00)	0.95 (0.93–0.97)	0.98 (0.970.-99)	0.98 (0.97–0.99)
Se[4]	0.89 (0.68–1.00)	0.89 (0.74–1.00)	0.94 (0.87–1.00)	0.50 (0.15–0.85)
Sp[4]	0.99 (0.98–1.00)	0.95 (0.93–0.97)	0.99 (0.98–1.00)	0.98 (0.97–0.99)
PPV[4]	0.53 (0.28–0.79)	0.36 (0.22–0.51)	0.85 (0.75–0.95)	0.31 (0.06–0.56)
NPV[4]	1.00 (0.99–1.00)	1.00 (0.99–1.00)	0.99 (0.99–1.00)	0.99 (0.99–1.00)
κ[4]	0.68 (0.49–0.88)	0.35 (0.19–0.50)	0.88 (0.80–0.95)	0.18 (0.00–0.40)
κ *P*-value[5]	0.095	<0.001	0.131	0.225

[1]Biplate with selective chromogenic culture media for gram-positive and gram-negative bacteria (Mastitis GP and Mastitis GN, respectively, both from CHROMagar).

[2]Total number of samples testing positive on the biplate.

[3]Total number of samples testing positive to the gold-standard test (traditional).

[4]Values in parentheses = 95% CI.

[5]Cohen's Kappa concordance test.

growth of colonies with a single morphology in 53% (253/476) of the samples, 4% (20/476) had 2 distinct morphologies, 2% (11/476) were contaminated, and 40% (192/476) of the samples had no microbial growth.

For the SCM samples, 60% (301/500) showed growth of colonies with a single morphology, 7% (37/500) had 2 morphologies, 1% (5/500) were contaminated, and 31% had no microbial growth. The prevalence of gram-positive bacteria was greater for *Staph. chromogenes* 29.4% (147/500) and other gram-positive bacteria 10.8 (54/500) in SCM samples. Among gram-negative bacteria, the prevalence was greater for *E. coli* 8.6% (41/476) and *Kleb. pneumoniae* 4.8% (23/476) in CM samples, and other gram-negative bacteria 2.6% (13/500) and *Kleb. pneumoniae* 0.8% (4/500) in SCM samples (Table 4).

Performance of Chromogenic Culture Media Triplates

The Ac of chromogenic culture media triplate in CM samples ranged from 0.93 (NAS) to 0.99 (*Pseudomonas* spp. and *Prototheca* spp. or yeast). On the other hand, the sensitivity of chromogenic culture media triplate

Table 4. Distribution of mastitis-causing microorganisms identified by microbiological milk culture according to standard MALDI-TOF MS methodology (CM = 476; SCM = 500), evaluated by triplate chromogenic culture media[1]

	CM		SCM	
Variable	No.	%	No.	%
Total samples	476	100	500	100
No growth	192	40.3	157	31.4
Colonies with 1 morphology	254	53.4	299	59.8
Colonies with 2 morphologies	19[2]	4.0	39[3]	7.8
Contamination	11	2.3	5	1.0
Gram-positive bacteria	155	32.6	279	55.8
Staphylococcus chromogenes	39	8.2	147	29.4
Staphylococcus aureus	27	5.7	32	6.4
Streptococcus uberis	16	3.4	7	1.4
Staphylococcus sciuri	12	2.5	1	0.2
Streptococcus agalactiae	10	2.1	3	0.6
Streptococcus dysgalactiae	9	1.9	7	1.4
Corynebacterium spp.	8	1.7	4	0.8
Enterococcus spp.	6	1.3	4	0.8
Lactococcus spp.	7	1.5	20	4.0
Other gram-positive bacteria	21[4]	4.4	54[5]	10.8
Gram-negative bacteria	88	18.5	20	4.0
Escherichia coli	41	8.6	1	0.2
Klebsiella pneumoniae	23	4.8	4	0.8
Serratia marcescens	10	2.1	0	0
Enterobacter cloacae	3	0.6	1	0.2
Klebsiella oxytoca	3	0.6	1	0.2
Other gram-negative bacteria	8[6]	1.7	13[7]	2.6
Other microorganisms	11	2.3	0	0
Candida tropicalis	2	0.4	0	0
Candida rugosa	5	1.1	0	0
Other yeasts	4	0.8	0	0

[1]Smartcolor2 (Onfarm).

[2]*Escherichia coli* and others (n = 1); *Enterobacter* and others (n = 1); *Lactococcus* spp. and NAS (n = 1); *Lactococcus* spp. and others (n = 2); NAS and NAS (n = 1); NAS and others (n = 3); Others and others (n = 1); *Staphylococcus aureus* and *Streptococcus dysgalactiae* (n = 1); *E. coli* and *Strep. dysgalactiae* (n = 1); *Strep. dysgalactiae* and NAS (n = 1); *Strep. dysgalactiae* and others (n = 1); *Strep. uberis* and *Klebsiella* spp. (n = 1); *Strep. uberis* and NAS (n = 4).

[3]*Enterobacter* spp. and others (n = 1); *Enterococcus* spp. and NAS (n = 3); *Lactococcus* spp. and NAS (n = 10); NAS and NAS (n = 6); NAS and others (n = 4); *Staph. aureus* and *Lactococcus* spp. (n = 2); *Staph. aureus* and NAS (n = 2); *Staph. aureus* and others (n = 2); *Staph. aureus* and *Strep. agalactiae* (n = 1); *Staph. aureus* and *Strep. uberis* (n = 2); *Strep. uberis* and NAS (n = 6).

[4]Mainly gram-positive genera: *Aerococcus* spp. (n = 2); *Bacillus* spp. (n = 2); NAS (n = 9); *Paenibacillus* spp. (n = 2).

[5]Mainly gram-positive genera: *Aerococcus* spp (n = 4); *Bacillus* spp. (n = 1); NAS (n = 44).

[6]Mainly gram-negative genera: *Citrobacter* spp. (n = 2); *Pseudomonas* spp. (n = 4).

[7]Mainly gram-negative genera: *Acinetobacter* spp. (n = 7); *Citrobacter* spp. (n = 1); *Pseudomonas* spp. (n = 4).

Table 5. Diagnostic accuracy (specificity, Sp; sensitivity; Se; accuracy, Ac; positive predictive value, PPV; negative predictive value, NPV; and Cohen's kappa agreement coefficient, κ) of the triplate[1] chromogenic media[2] in milk samples from cows with CM (n = 476)

Variable	*Streptococcus agalactiae* or *Streptococcus dysgalactiae*	*Streptococcus uberis*	*Staphylococcus aureus*	NAS	*Enterococcus* spp.	*Lactococcus* spp.
n[3]	48	25	37	57	16	15
n[4]	23	21	28	70	6	10
Ac[5]	0.93 (0.92–0.96)	0.98 (0.97–0.99)	0.96 (0.95–0.98)	0.92 (0.89–0.94)	0.96 (0.95–0.98)	0.96 (0.94–0.97)
Se[5]	0.87 (0.73–1.00)	0.86 (0.71–1.00)	0.86 (0.73–0.99)	0.63 (0.52–0.74)	0.43 (0.06–0.80)	0.20 (0.00–0.45)
Sp[5]	0.94 (0.92–0.96)	0.98 (0.97–1.00)	0.97 (0.96–0.99)	0.97 (0.95–0.99)	0.97 (0.96–0.99)	0.97 (0.95–0.98)
PPV[5]	0.42 (0.28–0.56)	0.72 (0.54–0.90)	0.65 (0.50–0.80)	0.77 (0.66–0.88)	0.19 (0.00–0.38)	0.13 (0.00–0.31)
NPV[5]	0.99 (0.99–1.00)	0.99 (0.99–1.00)	0.99 (0.98–1.00)	0.94 (0.91–0.96)	0.99 (0.98–1.00)	0.98 (0.97–0.99)
κ[5]	0.53 (0.39–0.68)	0.75(0.61–0.89)	0.70 (0.58–0.82)	0.71 (0.61–0.80)	0.12 (0.00–0.30)	0.12(0.00–0.29)
κ *P*-value[6]	<0.001	0.1317	0.371	<0.001	0.0007	0.1025

[1]Smartcolor2 (Onfarm).

[2]Triplate with chromogenic culture media (*Streptococcus*, *Staphylococcus*, and gram-negative).

[3]Total number of samples testing positive on the tri-plate.

[4]Total number of samples testing positive to the gold-standard test (traditional).

[5]Values in parentheses = 95% CI.

[6]Cohen's kappa concordance test.

ranged from 0.09 (*Serratia* spp.) to 0.94 (*Klebsiella* spp. and *Enterobacter* spp.), whereas the Sp ranged from 0.94 (*Strep. agalactiae* and *dysgalactiae*) to 1.00 (*Serratia* spp.).

Considering PPV, the results ranged from 0.13 (*Lactococcus* spp.) to 0.78 (*Prototheca* spp. and yeast.), whereas the NPV ranged from 0.94 (NAS) to 1.00 (*Klebsiella* spp. and *Enterobacter* spp.; *Prototheca* spp. or yeast; Tables 5 and 6).

In CM samples, agreement between the evaluated methodologies ranged from 0.07 to 0.85. Almost perfect agreement was observed for the identification of *Klebsiella* spp. and *Enterobacter* spp. (κ = 0.85), substantial agreement was observed for *E. coli* (κ = 0.69), *Staph. aureus* (κ = 0.70), NAS (κ = 0.71), *Strep. uberis* (κ = 0.75), and *Prototheca* spp. or yeast (κ = 0.78), moderate agreement for *Strep. agalactiae* and *dysgalactiae* (κ = 0.53), *Pseudomonas* spp. (κ = 0.60), and it was considered a slight agreement test for *Enterococcus* spp. (κ = 0.12), *Lactococcus* spp. (κ = 0.12), *Serratia* spp. (κ = 0.07; Tables 5 and 6).

The Ac of the triplate of chromogenic culture media in SCM samples ranged from 0.83 (NAS) to 1.00 (*Pseudomonas* spp.). The Se of the chromogenic culture media triplate ranged from 0.25 (*Lactococcus* spp.) to 1.00 (*Strep. agalactiae* and *dysgalactiae*), and the Sp

Table 6. Diagnostic accuracy (specificity, Sp; sensitivity; Se; accuracy, Ac; positive predictive value, PPV; negative predictive value, NPV; and Cohen's kappa agreement coefficient, κ) of the triplate[1] chromogenic media[2] in milk samples from cows with CM (n = 476)

Variable	*Escherichia coli*	KE[3]	*Serratia* spp.	*Prototheca* or yeast
n[4]	51	39	2	13
n[5]	43	32	11	10
Ac[6]	0.95 (0.93–0.97)	0.98 (0.96–0.99)	0.98 (0.96–0.99)	0.99 (0.98–1.00)
Se[6]	0.79 (0.67–0.91)	0.94 (0.85–1.00)	0.09 (0.00–0.26)	0.91 (0.74–1.00)
Sp[6]	0.96 (0.65–0.98)	0.98 (0.97–0.99)	1.00 (0.99–1.00)	0.99 (0.99–1.00)
PPV[6]	0.67 (0.54–0.80)	0.77 (0.64–0.90)	0.50 (0.00–1.00)	0.78 (0.54–1.00)
NPV[6]	0.98 (0.97–0.99)	1.00 (0.99–1.00)	0.98 (0.97–0.99)	1.00 (0.99–1.00)
κ[7]	0.69 (0.60–0.80)	0.85 (0.75–0.94)	0.07 (0.00–0.24)	0.78 (0.59–0.97)
κ *P*-value	0.079	0.317	0.025	0.654

[1]Smartcolor2 (Onfarm).

[2]Triplate with chromogenic culture media (*Streptococcus*, *Staphylococcus*, and gram-negative).

[3]KE = *Klebsiella* spp. or *Enterobacter* spp.

[4]Total number of samples testing positive on the tri-plate.

[5]Total number of samples testing positive to the gold-standard test (traditional).

[6]Values in parentheses = 95% CI.

[6]Cohen's kappa concordance test.

ranged from 0.92 (*Strep. agalactiae* and *dysgalactiae*; NAS) to 0.99 (*Lactococcus* spp. and *Klebsiella* spp. or *Enterobacter* spp.).

Considering PPV, the results ranged from 0.29 (*Enterococcus* spp.) to 0.88 (NAS), whereas NPV ranged from 0.81 (NAS) to 1.00 (*Klebsiella* spp. or *Enterobacter* spp. and *Strep. agalactiae* or *dysgalactiae*; sTable 7).

The agreement results between the standard methodology and the triplate chromogenic culture media in SCM samples ranged from 0.33 to 0.72. Moderate agreement was observed for the identification of *Strep. uberis* ($\kappa = 0.49$), *Klebsiella* spp. or *Enterobacter* spp. ($\kappa = 0.55$), substantial agreement for NAS ($\kappa = 0.62$), *Pseudomonas* spp. ($\kappa = 0.66$), and *Staph. aureus* ($\kappa = 0.72$), and it was considered a fair agreement test for the identification of *Enterococcus* spp. ($\kappa = 0.33$), *Strep. agalactiae* and *dysgalactiae* ($\kappa = 0.33$), *Lactococcus* spp. ($\kappa = 0.36$), and *E. coli* ($\kappa = 0.28$; Table 7).

DISCUSSION

Experiment 1

In the present study, the diagnostic performance of the biplate containing chromogenic culture media was evaluated for the rapid identification of the main microorganisms causing CM and SCM in milk samples. The microorganisms most isolated in CM samples were *Strep. uberis* and *E. coli*, whereas for SCM, *Staph. aureus* and NAS were the most isolated. In previous studies on the evaluation of mastitis diagnostic methods, a distribution of mastitis-causing agents similar to those of the present study was observed (McCarron et al., 2009; Royster et al., 2014; Viora et al., 2014; Ganda et al., 2016; Griffioen et al., 2018).

The use of the chromogenic media biplate resulted in high Se (>0.89) and Sp (>0.94) for the identification of *Strep. uberis*, both in the samples of CM and SCM. Likewise, the Se of identification of *Strep. agalactiae* and *Strep. dysgalactiae* ranged from 0.71 (CM) to 0.89 (SCM), whereas the Sp was >0.97 in samples of CM and SCM. The results of the present study are similar to those described for the identification of bacteria in the *Streptococcus* spp. group, using a triplate composed of culture media enriched with modified thallium sulfate crystal, with 0.93 Se and Sp. 0.90 (McCarron et al., 2009). Similarly, the use of AccuMast triplate to identify *Streptococcus* spp. presented Se = 0.90 and Sp = 0.93, although *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis* species were not differentiated (Ganda et al., 2016). The capacity of differentiation among *Streptococcus* spp. isolated in the biplate chromogenic media allows a more specific identification of group of pathogens or at the species level. In this case, rapid culture results could be used for segregation of cows with contagious transmission (e.g., *Strep. agalactiae* and *Strep. dysgalactiae*) and for extended clinical therapy (*Strep. uberis*). One of the limitations of using the GP chromogenic media is the lack of differentiation of *Strep. agalactiae* and *Strep. dysgalactiae* because they have similar colony color (light blue or turquoise). Additionally, the differentiation of *Lactococcus* spp. and *Enterococcus* spp. is not possible by the GP culture media. This limitation implies the need for additional biochemical tests for the differentiation between these species.

Table 7. Diagnostic performance (specificity, Sp; sensitivity; Se; accuracy, Ac; positive predictive value, PPV; negative predictive value, NPV; and Cohen's kappa agreement coefficient, κ) of triplate[1] chromogenic culture media[2] in milk samples cow with SCM (n = 500)

Variable	*Streptococcus agalactiae* or *Streptococcus dysgalactiae*	*Streptococcus uberis*	*Staphylococcus aureus*	NAS	*Enterococcus* spp.	*Lactococcus* spp.	*Klebsiella spp.* or *Enterobacter spp.*
n[3]	50	26	45	182	14	11	11
n[4]	11	15	41	215	7	32	7
Ac[5]	0.92 (0.90–0.95)	0.96 (0.95–0.98)	0.96 (0.95–0.98)	0.83 (0.80–0.87)	0.97 (0.96–0.99)	0.95 (0.930.-97)	0.98 (0.97–1.00)
Se[5]	1.00 (1.00–1.00)	0.73 (0.51–0.96)	0.83 (0.72–0.95)	0.72 (0.66–0.78)	0.50 (0.15–0.85)	0.25 (0.10–0.40)	0.71 (0.38–1.00)
Sp[5]	0.92 (0.90–0.94)	0.97 (0.95–0.98)	0.98 (0.96–0.99)	0.92 (0.90–0.95)	0.98 (0.97–0.99)	0.99 (0.99–1.00)	0.99 (0.98–1.00)
PPV[5]	0.22 (0.11–0.33)	0.42 (0.23–0.61)	0.76 (0.64–0.88)	0.88 (0.83–0.93)	0.29 (0.05–0.52)	0.73 (0.46–0.99)	0.45 (0.16–0.75)
NPV[5]	1.00 (1.00–1.00)	0.99 (0.98–1.00)	0.98 (0.97–1.00)	0.81 (0.76–0.85)	0.99 (0.98–1.00)	0.95 (0.93–0.97)	1.00 (0.99–1.00)
κ[6]	0.33 (0.18–0.48)	0.49 (0.30–0.68)	0.72 (0.60–0.82)	0.62 (0.55–0.69)	0.33 (0.08–0.59)	0.36 (0.17–0.54)	0.55 (0.27–0.83)
κ *P*-value	<0.0001	0.0046	0.2971	0.0008	0.0201	<0.0001	0.1573

[1]Smartcolor2 (Onfarm).

[2]Triplate with chromogenic culture media (*Streptococcus*, *Staphylococcus*, and gram-negative).

[3]Total number of samples testing positive on the tri-plate.

[4]Total number of samples testing positive to the gold-standard test (traditional).

[5]Values in parentheses = 95% CI.

[6]Cohen's kappa concordance test.

We observed a low Se (0.56) and PPV (0.64) for *Staph. aureus* identification using GP chromogenic media. Similar results for Se and PPV were described using the VétoRapidkit, the Se and PPV of which were 0.57 and 0.54, respectively, for SCM samples (Viora et al., 2014). In the present study, in 467 samples of CM, we observed 7 FN results for the identification of *Staph. aureus* in the GP media due to the growth of colonies with a different color than indicated (light pink). However, in the SCM samples, the GP culture media showed Se of 0.89 for identification of *Staph. aureus*. Similar to the results of the SCM samples, other studies on chromogenic culture media selective for *Staphylococcus* spp. reported Se of 1.0 and Sp of 1.0 of *Staph. aureus* identification (Ganda et al., 2016). Studies that evaluated the growth of *Staph. aureus* using Petrifilm reported Se of 92.1 and Sp of 0.93 (McCarron et al., 2009).

Regarding gram-negative bacteria isolated from CM samples, the GN chromogenic culture media showed Se of 0.89 (*E. coli*) and Sp of 0.97 (*E. coli*). In addition, in SCM samples, Se was 0.50 and Sp was 0.98 for identification of *E. coli*. Similar results have been described for the identification of *E. coli* using the AccuMast triplate, with Se = 1.0 and Sp = 0.97, and for *Klebsiella* spp., *Enterobacter* spp., or *Serratia* spp. of Se = 0.98 and Sp = 0.96 (Ferreira et al., 2018). In contrast, a lower Se (0.75) was reported for identification of *E. coli* in GN chromogenic culture media (Ganda et al., 2016). Gram-negative chromogenic media allow differentiation among the enterobacteria group, which is especially important for *Klebsiella* spp. and *E. coli*. This differentiation is critical because *E. coli* has a high rate of spontaneous cure without the use of antimicrobial treatment (Tomazi et al., 2018), whereas *Klebsiella* spp. may be associated with more severe and long-term mastitis cases that require treatment with antimicrobials (Fuenzalida and Ruegg, 2019). In our study, for CM samples, the identification of *Prototheca* spp. or yeast showed Se of 0.73 and Sp of 1.00, which indicates that this culture medium is useful for presumptive rapid identification of this group of pathogens. However, additional tests are needed to differentiate between the 2 groups.

The Ac for the identification of *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis* was >0.94 in the CM and SCM samples. Previous studies that evaluated the identification of the group of *Streptococcus* spp. did not describe the differentiation between species, which makes it difficult to compare the Ac values with those obtained in the present study. Thus, the accuracy of identification of *Streptococcus* spp. was 0.92 (Ganda et al., 2016) and 0.94 (Ferreira et al., 2018) with the use of the AccuMast triplate, 0.90 for the Minnesota Easy System, 0.93 for Mastitis SSGN Quad plate media (DQCI Services), and 0.89 for Mastitis SSGNC Quad plate (DQCI Services; Ferreira et al., 2018). Thus, the GP culture media gave similar results for accuracy compared with the other evaluated media. However, it was still the only one that differentiated the results of the groups of *Strep. agalactiae* or *Strep. dysgalactiae* and *Strep. uberis*. The Ac of *Staph. aureus* identification in the GP culture media was >0.97 in the CM and SCM samples, performance similar to that described by Ganda et al. (2016), who reported Ac of 1.0 with the use of the Triple AccuMast. The high Ac for this pathogen in our study may be associated with its low prevalence in CM (14/476) and SCM (43/660) samples. Regarding *E. coli*, identification accuracy was 0.97, similar to that described for the AccuMast culture media (0.96; Ganda et al., 2016) and the Mastitis SSGN Quad plate method (0.90; Ferreira et al., 2018). Regarding the group of *Klebsiella* spp., *Enterococcus* spp., and *Serratia* spp., the GN culture media showed an Ac of 0.99 in CM samples, similar to that reported by Ganda et al. (2016) with an Ac value of 0.98. In previous studies on the evaluation of selective culture media for gram-negative bacteria, there was no differentiation of species or groups, classifying the results only as the absence or presence of coliforms.

Our results of PPV for the identification of *Strep. uberis* (CM: 0.59 and SCM: 0.36) were similar to those described by the use of VétoRapid in SCM samples (PPV = 0.22), although, in CM samples, our PPV was lower than values reported by Viora et al. (2014; PPV = 0.81). Similar variation was observed for the identification of *E. coli*, which had greater PPV values compared with VétoRapid in CM (PPV = 0.70), but a lower value in SCM (PPV = 0.34). However, for *Staph. aureus* in both CM and SCM, PPV values for our biplate were greater than those for VétoRapid (CM = 0.58 and SCM = 0.53), although for CM, the PPV was lower than that obtained by Accumast (0.88; Ganda et al., 2016).

In our study, NPV results for both CM and SCM samples were ≥0.97. These high NPV values can be attributed to the low prevalence of some pathogens (e.g., *E. coli* in SCM). For *Staph. aureus* identification in CM, NPV was 0.98, which is similar to the results described for Accumast (1.00). Additionally, the NPV for *Strep. uberis* in both CM and SCM was higher than those reported by Viora et al., 2014 (0.98 for CM and SCM).

The agreement between the standard methodology and the GP media in CM cases was moderate for *Staph. aureus* ($\kappa = 0.47$) and substantial for *Strep. uberis* or *Enterococcus* spp. and *Strep. agalactiae* or *Strep. dysgalactiae* ($\kappa = 0.67$). In contrast, agreement was fair for *Strep. uberis* ($\kappa = 0.35$) and almost perfect for *Staph.*

aureus ($\kappa = 0.88$) in the SCM samples. In a recent study of 4 rapid diagnostic methods on the farm (CHROMagar Mastitis, Hardy Diagnostics Mastitis Triplate, Minnesota Easy Culture S-II Triplate, and VétoRapid), flawed agreement was observed between standard microbiological culture and CHROMagar chromogenic media and culture, mainly for gram-positive bacteria ($\kappa = 0.23$; Griffioen et al., 2018). For *Staph. aureus*, the kappa values obtained in the GP media for SCM ($\kappa = 0.88$) were similar to those described for AccuMast (Ganda et al., 2016), which showed almost perfect agreement ($\kappa = 0.93$) with the standard methodology. For the identification of gram-negative bacteria in CM samples, the agreement results for the GN media and the standard methodology was 0.84 for *E. coli*, indicating almost perfect agreement between the evaluated methodologies. This high agreement value was similar to the results described with the AccuMast culture media, with $\kappa = 0.81$ for gram-negative bacteria (Ferreira et al., 2018). For *Prototheca* spp. or yeast, the κ value was 0.80, which indicates substantial agreement.

Our results suggest that the diagnostic accuracy of the evaluated chromogenic culture media (GP and GN) varied according to the species of the pathogen (e.g., *E. coli, Klebsiella* spp., *Staph. aureus*, and *Strep. agalactiae* or *Strep. dysgalactiae*). The biplate had adequate accuracy for rapid presumptive identification of *Streptococcus* spp. (high Se and Sp of the groups *Strep. agalactiae* or *Strep. dysgalactiae* and *Strep. uberis*), and *Klebsiella* spp. (as observed on high Se and Sp, and high Sp for *E. coli*. The Se for *Staph. aureus* was low for CM (0.56) and high for SCM (0.94), and the Sp was high (CM = 0.99, SCM = 0.99). These results suggested the use biplate of chromogenic culture media depends on the demand of identification, and the situation on each farm (e.g., necessity of identification between *Staph. aureus* and NAS). The identification of mastitis-causing microorganisms were based on the incubation of the chromogenic culture media for 24 h, according to the manufacturer's recommendation; the characteristic coloration may change with a longer incubation period. Finally, we observed that the use of the GP culture media has limitations in the identification of *Staph. aureus*, due to the color variation presented by the colonies of these microorganisms.

Experiment 2

In the present study, the diagnostic performance of the triplate of chromogenic culture media was evaluated for the rapid identification of the main mastitis-causing microorganisms in milk samples. The microorganisms with the highest prevalence in CM samples were *Staph. chromogenes*, *Staph. aureus*, and *E. coli*, whereas in the SCM samples, *Staph. aureus* and *Staph. chromogenes* were the most isolated pathogens. Previous studies have also identified a high prevalence of NAS in milk samples from clinical and subclinical mastitis, using culture media for rapid identification (Viora et al., 2014; Griffioen et al., 2018). For example, a high prevalence of NAS and *Staph. aureus* was observed by use of biplates and triplates of the Minnesota Easy Culture system, as well as *Klebsiella* spp. in clinical and subclinical mastitis samples (Royster et al., 2014).

The Se results obtained by the triplate of chromogenic media for identification of *Strep. uberis* ranged from 0.73 in SCM samples to 0.86 in CM, whereas Sp results were 0.97 for SCM and 0.98 for CM. The use of the VétoRapid diagnostic kit (Viora et al., 2014) to identify *Strep. uberis* resulted in Se of 0.84 and Sp of 0.92, similar to values found in our study. Other studies have evaluated the performance of culture media for the identification of *Streptococcus* spp.; however, no species differentiation was done.

For the identification of *Enterococcus* spp., the Se ranged from 0.43 (CM) to 0.50 (SCM) and Sp was 0.97 for CM and 0.98 for SCM. The use of the AccuMast triplate to identify *Enterococcus* spp. resulted in Se = 0.88 and Sp = 0.95 (Ferreira et al., 2018). In contrast, the evaluation of the VétoRapidkit for the identification of *Enterococcus* spp. resulted in Se of 0.17 and Sp of 0.93 (Viora et al., 2014).

Regarding the identification of *Lactococcus* spp., we observed colony color variation according to the species (*Lactococcus garviae* and *Lactococcus lactis*). For example, *Lactococcus* spp. isolates should have a light purple (lilac) characteristic color, but *L. garviae* showed colonies with white or translucent color, which accounted for FN results of *Lactococcus* spp. group (n = 8 for CM and n = 25 for SCM). These results were also observed in a recent study, in which FN results were observed for the identification of *Lactococcus* spp. with the use of the Minnesota Easy System triple plate (Ferreira et al., 2018). This limitation in colony color variation negatively affected the Se results for the identification of *Lactococcus* spp. in the present study.

The Se and Sp results of the selective chromogenic media for *Staphylococcus* spp. varied according to the type of CM and SCM sample. The results for Se identification of *Staph. aureus* in the present study (Se = 0.86 to CM and 0.83 to SCM) were lower than those described by the Minnesota Triplate and 3M Petrifilm Staph Express system, which presented values of Se = 0.97 (McCarron et al., 2009) and AccuMast with Se = 1.00 (Ganda et al., 2016), but higher than those of the VétoRapid kit, which presented Se = 0.65 (Viora et al., 2014). Regarding Sp, the results of identification of *Staph. aureus* obtained in our study were superior to

those described by other studies, with Sp = 0.94 (Viora et al., 2014), 76.1% (McCarron et al., 2009) or similar Sp = 1.00; (Ganda et al., 2016). For the correct use of the results of rapid diagnostic tests, it is essential to obtain satisfactory results of Ac, Se, and Sp for the identification of *Staph. aureus*, differentiating it from other NAS species. False-negative results negatively affect the application of management measures such as segregation or culling of infected cows, in addition to the greater risk of transmission between cows (Royster et al., 2014).

For NAS identification, Se was 0.63 and Sp was 0.97 in CM samples, and Se was 0.72 and Sp was 0.95 in SCM samples. The identification of NAS presents variable results in other studies, with Se of 0.78 in the AccuMast triplate (Ganda et al., 2016), whereas for the VétoRapid kit, Se was 0.24 (Viora et al., 2014). In contrast, the Sp for NAS identification was similar to that of previous studies (Viora et al., 2014; Ganda et al., 2016). Control measures are different among the *Staphylococcus* group. Non-*aureus Staphylococcus* spp. have a high rate of spontaneous cure and are responsive to antimicrobial treatments (80–90%; Tomazi et al., 2015). However, *Staph. aureus* has a low cure rate after treatment (<25%), a low response to antimicrobials, and a high rate of transmission (Misra et al., 2018).

Recommended control measures for cows with *Staph. aureus* mastitis include the segregation and culling of infected cows and treating only primiparous cows and cows without a history of mastitis (Reksen et al., 2006). Based on our results, identification of positive *Staph. aureus* cows using chromogenic media should not be used as the only criteria for culling, because around 33% of the tested cows had false-positive results. Cows identified with *Staph. aureus* mastitis should be segregated from the herd and have samples collected for laboratory microbiological analysis. Therefore, rapid differentiation between *Staph. aureus* and NAS would help producers decide about specific management decisions on infected cows.

Regarding the group of gram-negative bacteria isolated, the Se for identification of *E. coli* ranged from 0.79 (CM) to 1.00 (SCM), whereas the Sp was >0.96. Variable results of Se and Sp for identification of *E. coli* have been described in other studies. The AccuMast triplate presented Se and Sp of 0.75 and 0.98 (Ganda et al., 2016) and 1.00 and 0.97 (Ferreira et al., 2018), whereas the VétoRapid kit presented Se and Sp of 0.58 and 0.98, respectively (Viora et al., 2014) and SSGNC culture media showed Se and Sp of 0.73 and 0.92 (Ferreira et al., 2018). For the identification of the *Klebsiella* spp. and *Enterobacter* spp. group, the Se and Sp results of the present study are similar to those described in the evaluation of the AccuMast triplate (Ganda et al., 2016), with Se = 0.98 and Sp = 0.97. In the present study, there were few isolations of *Serratia* spp. The results of Ac from the Smartcolor2 triplate were >0.95 for gram-negative bacteria and >0.92 for gram-positive bacteria, except for NAS (0.87) in SCM. The identification of *Staph. aureus* and the *Klebsiella* spp. or *Enterobacter* spp. had Ac of 0.96 to 0.98 for both CM and SCM samples, similar to the results obtained in previous studies (Ganda et al., 2016; Ferreira et al., 2018). Likewise, the Ac for the identification of *E. coli* was 0.95 for CM and 0.99 for SCM, whose results are similar to that described in other studies (Ganda et al., 2016; Ferreira et al., 2018).

The PPV results observed for *Strep. uberis* identification were slightly lower for CM (0.72) than those reported for the VétoRapid kit (0.81; Viora et al., 2014). We observed a low PPV for identification of *Strep. uberis* in SCM samples, which could be partially attributed to the low prevalence of this pathogen in the present study.

For *Staph. aureus*, PPV was 0.65 and 0.76 for CM and SCM, respectively, similar to those of previous studies, which had PPV of 0.49 (McCarron et al., 2009), 0.58 (CM), and 0.53 (SCM; Viora et al., 2014), but lower than the PPV (0.88) observed for Accumast (Ganda et al., 2016). The NPV varied from 0.87 to 1.00 among the microorganisms evaluated in CM and SCM samples in this study. In general, our study showed high NPV, which could be due to the low frequency of isolation of the pathogens.

The Cohen's kappa agreement results varied between the triplate identification and the standard methodology, according to the type of pathogen isolated. For gram-negative bacteria, agreement varied from $\kappa = 0.07$ for *Serratia* spp. and $\kappa = 0.85$ for *Klebsiella* spp. and *Enterobacter* spp. in CM. The highest values (almost perfect agreement; $\kappa = 0.80$–1.00) were observed only for *Klebsiella* spp. and *Enterobacter* spp. in the CM samples ($\kappa = 0.85$). Different results were found using the Minnesota Easy Culture System biplate and triplate results, for which agreement varied for both methods from $\kappa = 0.21$ to 0.50 for gram-negative bacteria (Royster et al., 2014). For the *Staphylococcus* selective media, agreement varied from moderate to substantial for *Staph. aureus* ($\kappa = 0.70$ for CM and $\kappa = 0.72$ for SCM) and NAS ($\kappa = 0.71$ for CM and $\kappa = 0.62$ for SCM). These values are lower than those described by Ganda et al. (2016), who obtained almost perfect agreement for the diagnosis of *Staph. aureus* ($\kappa = 0.93$) and moderate agreement for NAS ($\kappa = 0.52$).

Both experiments 1 and 2 have similar limitations, including the use of MALDI-TOF, an imperfect reference test, as the only "gold standard" for mastitis pathogen identification. The most critical limitation

of the MALDI-TOF methodology is that the manufacturer's spectral database is based mainly on clinical microbial isolates from humans. The lack of strains of pathogens isolated from cases of bovine mastitis could affect the ability to identify these microorganisms, due to variations in ribosomal protein expression. To overcome this limitation of MALDI-TOF methodology, it is possible to use expanded local libraries by including additional spectra of mastitis-causing pathogens (Nonnemann et al., 2019).

Additional limitation of both chromogenic media (biplate and triplate) is the inability to differentiate between *Strep. agalactiae* and *Strep. dysgalactiae* because both species present similar colony color pattern. This differentiation is important in both SCM and CM samples, because each pathogen may require different control measures. For example, cows with SCM caused by *Strep. agalactiae* are recommended to be treated with antimicrobials during lactation (Keefe, 2012), whereas for *Strep. dysgalactiae*, treatment using dry cow therapy would be more adequate, resulting in cure risk close to 100% (Whist et al., 2007; Freu et al., 2020). Regarding the diagnosis of cows with CM caused by *Strep. agalactiae* or *Strep. dysgalactiae*, the differentiation between these 2 species is also critical because the identification of positive *Strep. agalactiae* cows indicates the need to screen the whole herd for identification of other infected cows within the herd using laboratory microbiological identification.

Additional limitations of this study included the visual reading of bacteria colonies on the chromogenic media, which were carried out by only one researcher; however, this evaluator was only blinded to the gold standard results. The low frequency of isolation of specific mastitis pathogens (e.g., *Prototheca* spp., *Pseudomonas* spp.) also made it difficult to evaluate the diagnostic performance of chromogenic media for those pathogens. Finally, the chromogenic media were evaluated under laboratory conditions, not on-farm conditions, and therefore results of this study should be interpreted with caution for on-farm evaluations.

CONCLUSIONS

The diagnostic accuracy of the biplate and triplate of chromogenic culture media varied according to the type of microorganism causing bovine mastitis and by the mastitis presentation (clinical or subclinical). The results suggested adequate accuracy for identification of *Strep. agalactiae* or *Strep. dysgalactiae*, *Strep. uberis*, *E. coli*, and *Klebsiella* spp. or *Enterobacter* spp. For *Staph. aureus*, Se was adequate only for use of the chromogenic culture triplate. The use of these chromogenic culture media allows rapid identification (<24 h) of the main mastitis-causing pathogens, based on the color of the microbial colonies, which could be useful for quick decision-making on clinical mastitis treatment protocols, but their use for implementation of mastitis control measures will depend on each farm's specific needs.

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