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Research Article

Evaluation of a Chromogenic Medium for the Identification and Isolation of MRSA

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Abstract

Introduction: We sought to evaluate the screening ability of the chromogenic medium, HardyCHROM™ MRSA, for the identification of Methicillin Resistant *Staphylococcus aureus* (MRSA) from clinical specimens at 24 hours and 48 hours of incubation.

Materials and Methods: 100 *Staphylococcus aureus* isolates were obtained from 162 non-repetitive clinical specimens which included: pus aspirates, blood cultures, urine; ear, wound and vaginal swabs. Stool specimens were excluded due to the possible interference of commensals. Suspected isolates of *S. aureus* were confirmed using Mannitol Salt Agar (MSA) and the coagulase test.

Two methods were compared for the detection of MRSA: (1) The Reference method -Kirby-Bauer disk diffusion method with a 30 (µg) cefoxitin disk on Mueller Hinton Agar (MHA) and (2) The Test method – observation of HardyCHROM™ MRSA after 24 and 48 hours. For the Reference method, a zone diameter of ≤21 mm was an indication that the isolate was MRSA, while ≥22 mm was identified as Methicillin Sensitive *Staphylococcus aureus* (MSSA). Pink to magenta colonies on HardyCHROM™ MRSA were interpreted as MRSA. No growth after 48 hours was documented as MSSA.

Results: A total of 100 *Staphylococcus aureus* isolates, obtained from 162 non-repetitive clinical specimens were processed. The specimens were mainly pus aspirates (36%), blood cultures (27%) and wound swabs (13%). After 24 hours, the sensitivity, specificity, PPV and NPV of HardyCHROM™ MRSA were 96.9%, 5.6%, 64.6% and 50.0% respectively. At 48 hours, the sensitivity, specificity, PPV and NPV of HardyCHROM™ MRSA were 96.9%, 0%, 63.3% and 0%, respectively. Using McNemar's test, to compare the two tests, a significant difference (p value <0.05) was found at 24 hours and 48 hours incubation

Conclusions: We concluded that because of the high sensitivity of HardyCHROM™ MRSA, this medium would be effective in the screening of patients or staff to identify persons with an MRSA infection or those carrying MRSA.

Keywords: Methicillin Resistant; *Staphylococcus aureus*; Chromogenic Media; Screening; Clinical Specimens; Identification

Abbreviations

ATCC: American Type Culture Collection; CA-MRS: Community Acquired Methicillin-Resistant *Staphylococcus aureus;* CDC: Center for Disease Control; CLSI: Clinical Laboratory Standards Institute; FDA: Food and Drug Administration; FN: False Negative; FP: False Positive; HA-MRSA: Hospital Acquired Methicillin-Resistant *Staphylococcus aureus;* LTCF: Long Term Care Facilities; MHA: Mueller Hinton Agar; MRSA: Methicillin-Resistant *Staphylococcus aureus;* MSA: Mannitol Salt Agar; MSSA: Methicillin-Susceptible *Staphylococcus aureus;* NPV: Negative Predictive Value; PBPs: Penicillin-Binding Proteins; PCR: Polymerase Chain Reaction; PPV: Positive Predictive Value; SCC: Staphylococcal Cassette Chromosome; SCCmec: Staphylococcal Cassette Chromosome mec; TN: True Negative; TP: True Positive

Introduction

With Methicillin Resistant *Staphylococcus aureus* (MRSA) presenting a threat in hospitals, Long Term Care Facilities (LTCF) and communities alike and its increasing prevalence and transmission through animals, the need for rapid detection is now critical [1-3]. MRSA infections are associated with high morbidity and mortality and MRSA has been identified as one of the high-priority antibiotic-resistant pathogens by the World Health Organisation [4,5].

MRSA identification is relatively time-consuming and very labour-intensive, as an initial culture period of 18 to 24 hours, followed by an additional 18 to 24 hours for antibiotic susceptibility testing (at minimum) is needed [6]. A delay in detection may have serious implications since it allows for the spread of MRSA if patients are not isolated [7]. In addition, delays in MRSA detection can impede treatment of patients, extend hospital stays, increase expenses for both hospitals and patients, and increase mortality rates [8].

Literature on methods for the detection and identification of MRSA in Guyana is lacking; however, it has been observed that traditional culture-based methods are used. The identification of MRSA involves initial culturing on culture media, Gram staining, followed by enzyme testing for the confirmation of *S. aureus*. Antibiotic susceptibility testing is then performed to determine antibiotic resistance.

Early, presumptive identification of MRSA from clinical samples affords the opportunity to be proactive in reducing the incidence and prevalence of Hospital Acquired MRSA (HA-MRSA) [9]. In addition, epidemiological studies can be conducted when surveying for the prevalence of Community Acquired MRSA (CA-MRSA). Although the Polymerase Chain Reaction (PCR) test method is the gold standard used for the detection of MRSA, the cost is prohibitive for some laboratories [10].

In other studies, the use of cefoxitin instead of oxacillin in selected media was recommended for the isolation of *S. aureus* isolates and screening using cefoxitin is recommended instead of PCR in institutions which have financial constraints [11,12].

Previous studies on chromogenic media have mainly focused on their use as screening tools with nasal swabs [13]. Chromogenic media offer a high degree of sensitivity and specificity and have been described as less costly and less time-consuming than traditional culture methods [6,14]. This allows for prompt decisions regarding the management of colonised patients. However, the changing epidemiology of MRSA, as new strains emerge, means that the suitability of these chromogenic media requires investigation [15]. An ideal chromogenic medium allows a definitive identification of microorganisms directly from the primary isolation plate, eliminating further subcultures and biochemical confirmatory tests [16].

This research team investigated the cost and availability of several chromogenic media and found that HardyCHROM™ MRSA was more readily available and affordable. Furthermore, HardyCHROM™ MRSA had been validated for isolation and identification of MRSA from nasal samples obtained from patients and healthcare workers but had not been used for the direct detection of MRSA from clinical specimens [17].

Research on the efficiency and efficacy of a brand of chromogenic medium for clinical samples that is both reasonably priced and easily accessible in Guyana, is necessary. This could subsequently lead to a template for laboratory diagnosis in public and private health care institutions and for epidemiological surveys, both in Guyana and throughout the Caribbean.

The main purpose of this study was to evaluate the screening ability of the chromogenic medium HardyCHROM™ MRSA for the isolation and identification of MRSA from clinical specimens at 24 hours and 48 hours of incubation.

Materials and Methods

This was an experimental study and recovery of MRSA on HardyCHROM™ MRSA was confirmed by the Kirby-Bauer disk diffusion method using a 30µg cefoxitin disk on Mueller-Hinton Agar (MHA) which served as the Reference method for this study.

The specific objectives of this study were:

- To determine the sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of HardyCHROM™ MRSA at 24 hours and 48 hours of incubation using clinical specimens.
- To determine whether a significant difference exists between HardyCHROM™ MRSA when compared to Kirby-Bauer disk diffusion method using a 30µg cefoxitin disk on Mueller Hinton for the detection of MRSA using different clinical specimens at 24 and 48 hours incubation.

Samples were collected from patients from four medical institutions within Georgetown, with suspected *S. aureus* infections from May-August, 2018. A total of 100 *S. aureus* isolates were obtained from 162 non-repetitive clinical specimens. Clinical specimens included: urine, ear swabs, wound swabs, blood cultures, pus aspirate, and vaginal swabs. Stool specimens were excluded due to the possible interference of commensals and coliforms and the influence of higher bacteria load. All specimens were processed within 24 hours after collection.

Quality control testing was successfully performed on each lot of HardyCHROM™ MRSA, Mueller Hinton agar with cefoxitin disk, and Mannitol Salt Agar (MSA) before use in the study. This was done using a standardised inoculum of American Type Culture Collection (ATCC) Methicillin Resistant *Staphylococcus aureus* 43300 (positive control strain) and ATCC Methicillin Susceptible *Staphylococcus aureus* (MSSA) 25923 (negative control strain).

Specimens were inoculated on MSA plates, incubated at 37°C for 24 hours, and examined for growth of *S. aureus* seen as yellow colonies. These colonies were then tested for coagulase activity by the tube coagulase test method using rabbit plasma.

For the detection of MRSA, two methods were used – The Reference method and the Test method:

- The Reference method – The Kirby-Bauer disk diffusion method was performed for all *S. aureus* isolates using the Clinical Laboratory Standards Institutes (CLSI) guidelines [18]; Individual colonies from MSA were obtained and a suspension made in saline to match a 0.5 McFarland standard; this was then introduced to the Mueller Hinton Agar (MHA) plate and a 30 (µg) cefoxitin disk was then applied. All plates were incubated at 35 ± 2°C. According to CLSI guidelines, a zone diameter of ≤21 mm was considered as MRSA, while ≥22 mm was reported as MSSA.
- The Test method - *S. aureus* isolates were inoculated on HardyCHROM™ MRSA using aseptic techniques and examined after 24 and 48 hours. All plates were incubated at 35 ± 2°C.

All 100 clinical specimens containing *S. aureus* isolates were inoculated directly onto HardyCHROM™ MRSA. The plates were kept in a dark incubator for 24 hours at 35 ± 2°C, without carbon dioxide (CO_2) and examined at 24 hours. Plates with no observed colony growth at the 24-hour point were re-incubated for an additional 24 hours and re-inspected for the presence of pink to magenta colonies. If no growth was observed after 48 hours of incubation, this was interpreted as MSSA. In this study, suspected pink to magenta colonies were interpreted as a positive result for MRSA (Figure 1).

Figure 1: HardyCHROM™ MRSA demonstrating pink to magenta colonies of MRSA.

True Positive (TP) was defined as a positive result on HardyCHROM™ MRSA, which correlated with a positive result by the Reference method. Similarly, a True Negative (TN) was defined as a negative result on HardyCHROM™ MRSA, which correlated with a negative result on the Reference method. A False Positive (FP) and False Negative (FN) were defined as positive and negative results, respectively, detected by HardyCHROM™ MRSA, which differed from the results seen in the Reference method.

Measures	Kirby-Bauer disk diffusion method using a 30 (µg) cefoxitin disk on Mueller-Hinton agar	HardyCHROM™ MRSA	Interpretation
TP	Zone of inhibition ≤21 mm	Pink to Magenta colonies	MRSA
FP	Zone of inhibition ≥22 mm	Pink to Magenta colonies	Discordant result
TN	Zone of inhibition ≥22 mm	No Growth	MSSA
FN	Zone of inhibition ≤21 mm	No Pink to Magenta colonies	Discordant result

Table 1: Interpretation of Results.

The interpretation of True Positives, True Negatives, False Positives and False Negatives are shown below (Table 1). A p value of <0.05 was considered statistically significant using the McNemar's test.

	Kirby-Bauer disk diffusion method using a 30µg cefoxitin disk on Mueller-Hinton agar			
		(+)	(-)	Measures
HardyCHROM™ MRSA	(+)	True positive	False positive	
	(-)	False negative	True negative	
Measures				

Table 2: Parameters and Formulae for Calculations.

The data were analysed using Statistical Package for Social Sciences (SPSS) software version 23. The sensitivity, specificity, positive and negative predictive value were calculated for HardyCHROM™ MRSA at 24 and 48 hours of incubation (Table 2).

Statistical significance was determined by the McNemar's test. A p value of <0.05 was considered statistically significant.

The following statistical formulae were used.

$$Sensitivity = \frac{TP}{TP + FN}$$

$$Specificity = \frac{TN}{TN + FP}$$

$$Positive\ Predictive\ Value\ (PPV) = \frac{TP}{TP + FP}$$

$$Negative\ Predictive\ Value\ (NPV) = \frac{TN}{TN + FN}$$

Results and Discussion

A total of 100 *Staphylococcus aureus* isolates, obtained from 162 non-repetitive clinical specimens between May 2018 and August 2018 were included in this study. The clinical specimens comprised of pus aspirates (36%), blood cultures (27%), wound swabs (13%), throat swabs (4%), skin scrapings (4%), vaginal swabs (3%), urine (2%), ear swabs (2%), nasal swabs (2%), sputa (2%) and other body sites (5%) (Figure 2).

At 24 hours of incubation, the Kirby-Bauer disk diffusion method with 30 µg cefoxitin disk on Mueller Hinton identified MSSA in 36 clinical specimens; whereas 4 specimens were identified as containing MSSA (that is, no growth was observed) using the

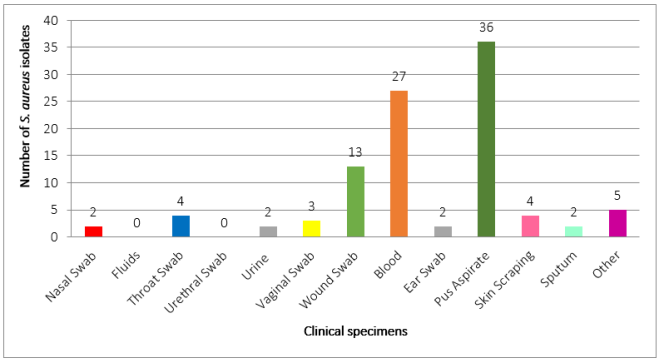

Figure 2: Distribution of *S. aureus* isolates from various clinical specimens.

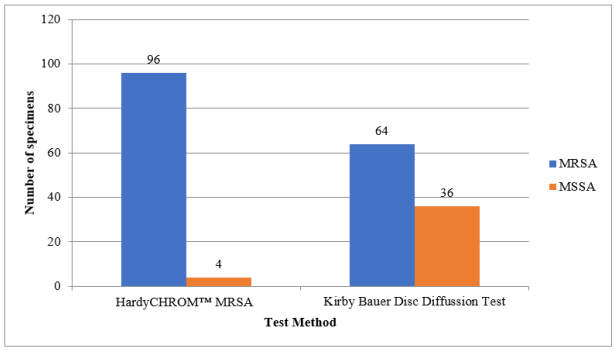

Figure 3: Number of MRSA and MSSA Identified by the Kirby Bauer Disk Diffusion Method and HardyCHROM™ MRSA at 24 hours.

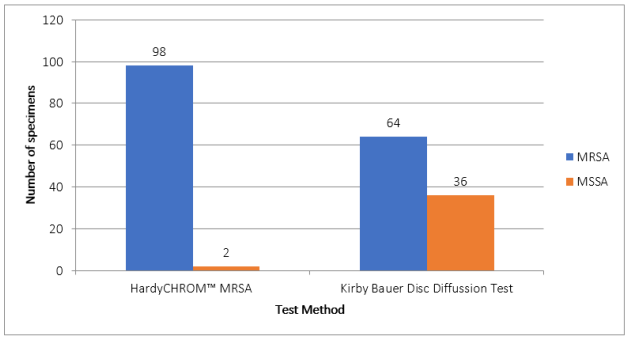

Figure 4: Number of MRSA and MSSA identified by the Kirby Bauer Disk Diffusion Method and HardyCHROM ™ MRSA at 48 hours.

HardyCHROM™ MRSA. Specimens identified as containing MRSA at 24 hours of incubation using the Kirby-Bauer disk diffusion method and the HardyCHROM™ MRSA were 64 and 96 specimens respectively. At 48 hours, the results were 64 and 98 specimens respectively (Figures 3-4).

Based on the comparison of results from Kirby-Bauer disk diffusion method using a 30 µg cefoxitin disk on Mueller-Hinton agar and HardyCHROM™ at 24 hours of incubation, there were 62 TP, 34 FP, 2 TN and 2 FN. Therefore, the sensitivity, specificity, PPV and NPV of HardyCHROM™ MRSA were 96.9%, 5.6%, 64.6% and 50.0% respectively. At 48 hours of incubation, there were 62 TP, 36 FP, 0 TN and 2 FN; hence the sensitivity, specificity, PPV and NPV were 96.9%, 0%, 63.3% and 0%, respectively (Tables 3 and 4).

Medium	Incubation time	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
HardyCHROM™ MRSA	24 hr	96.9	5.6	64.6	50.0
	48 hr	96.9	0.0	63.30	0.00

Table 3: Sensitivity, Specificity, PPV, and NPV of HardyCHROM ™ MRSA at 24 and 48 hours of incubation.

			Kirby-Bauer disk diffusion method using a 30µg cefoxitin disk on Mueller-Hinton agar			
			+	-	Total	p-value
HardyCHROM™ MRSA	24h	+	62	34	96	0
		-	2	2	4	
		Total	64	36	100	
	48h	+	62	36	98	0
		-	2	0	2	
		Total	64	36	100	

Table 4: Comparison of results between Reference method and Test method.

Traditional methods used for MRSA detection are time-consuming (3-4 days) and utilise more resources. Such delays in detection can have serious clinical and financial implications. Therefore, the introduction of an alternative, rapid and effective approach for the detection and identification of MRSA would be most beneficial. The high sensitivity of HardyCHROM™ MRSA at 24 and 48 hours, is an indication that the majority of patients with MRSA were detected when the medium was utilised as a screening method. Therefore, this medium could be effective in the preliminary identification of patients with an MRSA infection or to identify staff who may be carrying the bacterium.

In contrast, the specificity of HardyCHROM™ MRSA at 24 and 48 hours was poor. This meant that majority of patients were misdiagnosed as having MRSA when in reality, they did not.

Misdiagnosing patients with MRSA will result in greater economic burden on the patients and health institution [8]. Additionally, caring for misdiagnosed patients may result in the inefficient use of time and resources; these assets could be better utilised in other vital areas.

Furthermore, results for the PPV were comparable at 24 and 48 hours. A practical interpretation of the PPV meant that out of every 100 positive results obtained, approximately 63-64% would be accurate. It is known that there is relationship between PPV, specificity and prevalence. A greater prevalence results in a high PPV. When the prevalence is low, a greater specificity is needed to achieve a higher PPV; this means that a rare disease requires a test with a high specificity in order to correctly identify persons who do not have the disease [19]. The PPV for this research was computed without reference to the prevalence of MRSA in Guyana, as such there was difficulty in estimating its significance.

In contrast, the NPV decreased from 50.0% at 24 hours to 0% at 48 hours. This meant that at 24 hours, out of 100 negative results obtained, only 50% of patients did not have the disease; in contrast, at 48 hours, all patient results were negative when in reality everyone had the disease, which led to an NPV of 0%. Similar to PPV, there is also a relationship between NPV, sensitivity, and prevalence; NPV is greater when prevalence is low. A high prevalence requires a test with greater sensitivity to achieve a higher NPV; this means that highly prevalent disease requires a test with a high sensitivity in order to accurately identify persons who have the disease [19].

Using the McNemar's test, a p value <0.05 was obtained for the results of this study. This indicates that there was a significant difference between HardyCHROM™ MRSA and the Reference method at 24 and 48 hours incubation (Table 4).

Based on the trend of increasing prevalence of MRSA in the Caribbean, a test with a high sensitivity and PPV would be best suited for the initial detection and identification MRSA positive patients [20-22]. Furthermore, studies in Brazil, which shares a border with Guyana, have identified new and existing strains that are presenting challenges for effective therapy [23]. With the advent of development of the oil and gas sector in Guyana, it is therefore imperative to develop feasible and effective ways of quickly detecting and treating MRSA and other resistant pathogens.

A total of 34 isolates from the 96 specimens that yielded pink to magenta colonies at 24 hours were not MRSA according to results from the Kirby-Bauer disk diffusion method using a 30 µg cefoxitin disk on Mueller-Hinton agar; hence, these were false positives. When no MRSA was detected at 24 hours of incubation in two specimens, a longer incubation period led to the growth of MRSA isolates, increasing the number of false positives. A total of 36 false positive results were reported at 48 hours (36 isolates from 98 specimens). This suggests that prolonged incubation of HardyCHROM™ MRSA plates result in more false positives. Prolonging incubation time to 48 hours can improve sensitivities; however specificities are adversely affected, necessitating confirmatory tests before reporting MRSA [16].

Additionally, the false positive results may be due to the inherent formulation of the HardyCHROM™ MRSA. Various chromogenic media currently in use differ in their antibiotic formulations, chromogenic substrates, and/or concentrations and these are factors that impact their sensitivity and specificity for MRSA detection [16]. As such, it is difficult to postulate on the reason, as the chromogenic mixture, inhibitory and selective agents are not disclosed by the manufacturer.

Data were not collected to determine if any of the patients had other bacterial infections. Therefore, in the event of a mixed (polymicrobial) infection, the accuracy of HardyCHROM™ MRSA for detecting MRSA in the presence of other bacteria at a concentration of higher than 1×10^9 has not been determined and therefore is unknown [17]. Moreover, it is speculated that the possibility of other organisms in clinical specimens could have led to the hydrolysis of chromogenic substrates present in the media, eliciting the same colour change reaction which was initially only intended for MRSA from samples taken from the nares. During this research, clinical specimens from various body sites were used, therefore, increasing the likelihood of encountering other microbes, apart from *S. aureus*, which perhaps could have led to similar colour changes.

Another possible explanation for the large number of false positives may be due to variability among strains of MRSA, the presence of various *Staphylococcus species*. Researchers believe that one reason for the high number of false positive results is

the presence of heteroresistant or genetically varied strains of MRSA. CDC defines heteroresistance as the phenomena where two subpopulations (one susceptible and one resistant) coexist; that is, all cells may possess the gene for resistance, but very few cells may express it *in vitro*. Heteroresistant cells tend to grow slower than oxacillin susceptible populations and seldom grow at temperatures above 35°C [24].

Authors also described the expression of methicillin resistance in MRSA strains as homogenous, heterogenous and thermosensitive [25]. Heterogenous and heterogenous thermosensitive MRSA strains were able to synthesise the penicillin binding protein 2a (PBP2a); however, the ability to synthesis PBP2a alone was not enough to confer methicillin resistance. Heterogenous thermosensitive MRSA strains were methicillin resistant at 30°C but susceptible (MSSA) at 37°C [25], so it is possible that some of the strains we encountered were heterogenous thermosensitive and gave a false positive result.

It is well known that the *mecA* gene codes for methicillin resistance in MRSA; however, as recent as 2011, a variant of the *mec* gene (*mecC*) was identified and reported in both animals and humans [11,26]. Various studies have been conducted on the *mecA* homologue ($mecA_{LGA251}$ or *mecC*) found on the Staphylococcal Cassette Chromosome (SCC), which is 70% genetically identical to *mecA* and encodes a penicillin binding protein (PBP2c) that is 63% identical to the *mecA* PBP2a [27,28]. It was found that *mecC* strains of *S. aureus* have considerably reduced oxacillin and cefoxitin minimum inhibitory concentrations (MIC) when compared to *mecA S. aureus* strains [27,28]. This means that *mecC* strains of *S. aureus* can appear as MSSA at lower concentrations of oxacillin and cefoxitin than *mecA* strains of *S. aureus*. In this study, the concentration of the cefoxitin disk (30 µg) used as part of the Reference method may have been strong enough to cause *mecC* strains of *S. aureus* to appear as MSSA; however, the concentration of antibiotic in the chromogenic medium is not known, but we postulate that it was not sufficient to inhibit the growth of *mecC* strains of *S. aureus*.

With a total of 48 hours incubation, HardyCHROM™ MRSA failed to identify two MRSA positive specimens due to the absence of growth; these two results were recorded as false negatives. The failure may be explained by the quality of the specimens. We could not control how the specimens were collected, nor processed prior to receiving them from the clinical institutions and it is possible that the bacterial load in the specimens may have been reduced.

This study was limited with respect to the specimen collection and variation in specimen quality. The clinical specimens were obtained from four different institutions and therefore the researchers were unable to control the quality of the specimens collected. In addition, there was no way of knowing how the existence of polymicrobials in a sample, that is, both Gram negative and Gram positive, affected the enzymatic substrates within the chromogenic media in question. PCR testing is the preferred Reference method to compare the positive results observed from the chromogenic medium. However, our study utilised the Kirby-Bauer disk diffusion method using a 30 µg cefoxitin disk on Mueller-Hinton as the Reference method since DNA-based assays were neither affordable nor available at the time and this was another limitation.

We recommend that confirmatory tests such as latex agglutination should be used for the specific detection of the product of the *mecA* gene, penicillin-binding protein 2a, for confirmation of pink to magenta colonies; these tests will help to exclude or explain the presence of a false positive result. Moreover, any research to further analyse the performance of the chromogenic media should be done using PCR based assays as the Reference method, once it is economically feasible. In addition, when evaluating the chromogenic media with clinical specimens, we advocate that the specimens should be processed immediately upon collection and should only be used for the sole purpose of a study and should not undergo prior testing to ensure the availability of an adequate bacterial load.

Conclusion

To our knowledge, this is the first study designed to evaluate the performance of HardyCHROM™ MRSA at 24 and 48 hours incubation using various clinical specimens including nasal swabs used to validate the media. The chromogenic medium showed a high sensitivity and low specificity, and we suggest that it be considered for use when screening patients and staff in hospitals and Long Term Care Facilities, to determine if they are infected with and/or carrying MRSA.

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