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Research Article
Establishment and Application of the Resazurin Micro-plate Method for Rapid Diagnosis of Bovine Mastitis-causing Staphylococci

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Abstract

Background and Objective: Staphylococci strains causing bovine mastitis, has produced serious therapeutic problems. However, the underlying mechanisms are poorly understood and it is necessary to develop new effective method for accurate diagnosis. Resazurin micro-plate method was used for the diagnosis of bovine mastitis caused by staphylococci. The objective of this study was to discuss about the method of resazurin micro-plate for the diagnosing of bovine mastitis which caused by staphylococci. **Methodology:** While for testing the infected mastitis milk samples were inoculated in the selection of the Staphylococcus-mBP medium with 96-well micro-plate and pure cultures of bacteria and after 2 h of the incubation the resazurin indicator was added to that solution up to 0.4 g L^{-1} and then continued the cultivation for 10 h, depending on whether the medium color changes of bacterial growth. For statistical analysis chi-square test was performed using SPSS Software version 22 (IBM SPSS Statistics for Windows, Armonk, NY, USA: IBM Corp.). **Results:** The results from the bacterial pure culture plate showed that the resazurin micro-plate indicated the growth of *S. aureus* i.e., *S. epidermidis, S. chromogens, S. haemolyticus, S. simulans*, *S. xylosus* and *E. coli* did not appear on the culture plates. Further from the investigated results showed that the color changing in the medium was due to the staphylococcal concentration and time incubation. However, the results of the infection milk samples showed that the resazurin micro-plate had a detection sensitivity of 1×10^3 CFU mL^{-1} and in 12 h the diagnosis could be obtained. **Conclusion:** Furthermore from the results of the mastitis milk samples, the positive culture plate showed that the resazurin micro-diagnosis of *S. aureus*-positive and negative milk sample at the rate of 98.3% (n = 60) and 95.0% (n = 40) respectively. Similarly, from all the investigated results it is concluded that the resazurin micro-plate can replace the conventional method for rapid diagnosis of *Staphylococcus* milk samples.

Key words: Bovine mastitis, *Staphylococcus* resazurin micro-plate assay, diagnosis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Staphylococcus aureus is a Gram-positive spherical bacterium approximately 1 μm in diameter. Its cells form grape-like clusters since cell division takes place in more than one plane. It is often found as a commensal associated with skin, skin glands and mucous membranes particularly in the nose of healthy individuals. Mastitis is one of the most important diseases in dairy cows but not only caused serious economic losses and also there are public health risks[1], worldwide annual direct economic losses of mastitis of up to 82~131$ per head[2]. At present, the main dairy cow mastitis with antibiotics to control but due to lack of early diagnosis method for fast, accurate, long-term, blind use of antibiotics leads to drug-resistant strains of prevalent[3]. Although up to more than 150 species of pathogenic bacteria of dairy cow mastitis but mainly by *Staphylococcus, Streptococcus* and *E. coli*[4].

Staphylococci, including the *S. aureus* and *S. coagulase*-negative the including *S. aureus* which is resistant to all antibiotics[5]; while *S. coagulase*-negative were generally considered to be of environmental pathogens but in recent years has become an important pathogenic bacteria of dairy cow mastitis in many countries, mainly *S. epidermidis, S. aureus S. haemolyticus, S. stimulants and S. xylose*[6]. Bacterial culture has been the gold standard for identification of *S. aureus* in milk but it will take about a week to get results with less accurate and highly cost[3]. Represented by the PCR method in molecular biology has the advantages of rapid, sensitive but requires appropriate equipment and technical conditions and diagnosis is often confirmed the need to use conventional methods[7].

Previously developed research study conducted that mBP is a strong selective media for *Staphylococcus* while does not support the growth of bacteria's i.e., *Salmonellae, E. coli, Bacillus subtilis, Streptococcus, proteus, Pasteurella, Pseudomonas aeruginosa* bacteria, *Shigella* and *Staphylococcus*[8,9]. The media used of *S. coagulase*-positive restore the principles of potassium telluride which depending on whether or not black products judged and to have no growth of *S. aureus, S. coagulase* positive, so it cannot be used for rapid diagnosis of *S. coagulase*-negative. This study added to the mBP medium resazurin indicator established micro-plate method cannot only improve the efficiency of *S. aureus* mastitis diagnosis but can be used for rapid diagnosis of *S. coagulase*-negative.

MATERIALS AND METHODS

X-radiation dose rates were determined for a Polaris Model XR160 cabinet irradiator (Kimtron) using Fricke's dosimetry solution (0.8 N of H_2SO_4, 1 mM of $FeSO_4{\cdot}7H_2O$ and 1 mM of NaCl)[5]. Fricke's solution was placed in a 96-well microtiter plate (Fisher brand round bottom, Fisher Scientific) at 200 μL per well and irradiated for 3 min at the center of a metal platform 14 cm below a 3000 W, varian NDI-161 tube running at 160 kV and 15 mA. The dose rate in each well was determined by measuring the absorbance at 304 nm using a bio photometer. The mean dose rate over the 96 wells of triplicate plates was calculated from (ΔA304) (280 Gy min^{-1}) for 160 kV x-rays to be 17.96 Gy min^{-1} (sd = 0.02) (Fig. 1).

Strain, culture media and reagents: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylose*,

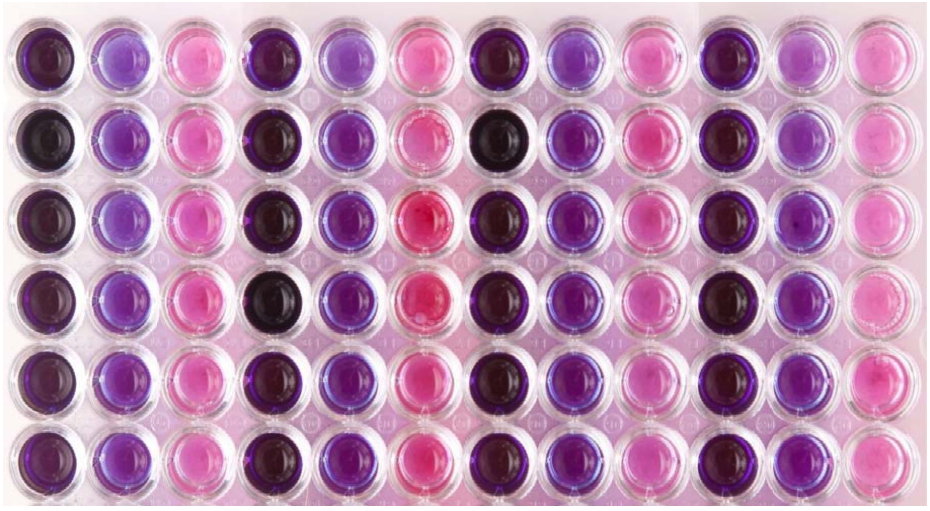

Fig. 1: Microtiter plate with three *E. coli* strains to demonstrate differential resazurin color change post X-irradiation

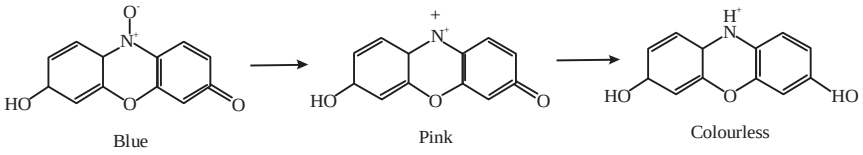

Fig. 2: Changes of resazurin and resulting color changes

Staphylococcus aureus, *Staphylococcus simulans*, *Hemolysis staphylococcus*, *Streptococcus agalactiae*, *dysgalactiae*, *uberis* and *E. coli* by our group identification and preservation[10]. Baird-Parker medium, MHB MacConkey media and media were purchased from Biological Technology Co., Ltd. 50 mL L^{-1} Sheep blood agar plates prepared according to standard methods. The mBP medium developed by our group, specific formulations patented authorization (200610038812.0). In mBP adding media 0.4 gL^{-1} resazurin sodium called medium mBP-RS Train Support groups. *Streptococcus* selective medium EN developed by the research group, specific formulations have been granted patent (Patent No. 200610038811.6). Other reagents were analytical grade.

Determination of the concentration of bacteria: Take *Staphylococcus aureus*, *Staphylococcus epidermidis, Streptococcus agalactiae*, *uberis* and each *E. coli* 1 plant, inoculate (*Streptococcus*) and free 50 g L^{-1} calf serum (Other bacteria) of LB agar plates 37°C incubated overnight, individual colonies were picked inoculated 5 mL LB medium 37°C to cultivate D600 nm = 1.0, pleasing 100 µL applying the corresponding agar plates 37°C to cultivate 24 h, colony counting counter, calculate D600 nm = 1.0 bacterial culture corresponding to the number of colonies and colony forming units (CFU L^{-1}) (Fig. 2).

Isolation and identification of bacteria in milk sampling: According to the results of the clinical examination and the detection of milk somatic cells, positive mastitis milk samples collected in accordance with aseptic manipulation, taking 1 mL with an equal amount PBS Mix, taking 200 µL blood agar plates were coated, Baird-Parker agar plates, EN agar plates and MacConkey agar plates 37°C to cultivate 24 h were picked *Streptococcus aureus*, *E. coli* colonies suspicious, Preparation of bacterial smears Gram staining, according to standard methods[11]. Biochemical tests, *Staphylococcus aureus* infection in the mammary gland of criteria $\geq$50 CFU mL^{-1}, coagulase-negative staphylococci and other bacterial infections criteria for breast $\geq$250 CFU mL^{-1}, cultures only a kind of single infection determined to colonies, produce two or more mixed infection determined to colonies[12].

Selective cultivation characteristics: Take *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylose*, *Staphylococcus aureus*, *Staphylococcus simulans*, *Hemolysis staphylococcus*, *dysgalactiae*, *Streptococcus agalactiae*, *uberis* and *E. coli* each 1 plant and they were inoculated with or without 50 mL L^{-1} calf serum LB medium 37°C to cultivate D600 nm = 1.0, according to D600 nm = 1.0 colony counting result of bacterial cultures, use PBS were diluted 1×10^2, 1×10^3, 1×10^4 with 1×10^5 CFU mL^{-1} taking 1 mL, 12000 r min^{-1} centrifugal 10 min, Bacterial pellet with 190 µL without indicator mBP medium suspension, packaging to 96 well culture plate 37°C to cultivate 2 h, each added 8 g L^{-1} resazurin sodium 10 µL, 37°C to cultivate 12 h. Depending on whether the medium color changes from blue to purple (Pink) red to determine the bacteria growth, in symbol form i.e. Slight discoloration found (+), moderate discoloration found (++), purple condemned (+++), pink found (++++) and the medium color ($\geq$++) outsourcing positive for bacterial growth.

Dynamic observation of the growth *Staphylococcus aureus*: Take *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylose*, *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Staphylococcus simulans* cultures of each 1 mL, bacteria were pelleted by centrifugation as before. According to D600 nm = 1.0 *Staphylococcus* colony counting result culture, use PBS were diluted 1×10^2, 1×10^3, 1×10^4 with 1×10^5 CFU mL^{-1}. Taking 1 mL centrifugal, bacteria were pelleted by 190 µL without indicator mBP medium suspension, as observed before and cultured.

Bacterial infection simulation training observation of milk samples: From each of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *dysgalactiae*, *Streptococcus agalactiæ*, *Streptococcus uberis* and *E. coli* cultures 1 mL, bacteria were pelleted by centrifugation; pasteurized milk is diluted with 1×10^3, 1×10^4, 1×10^5 with 1×10^6 CFU mL^{-1}, taking 1 mL, 12000 r min^{-1} centrifugal 10 min bacterial pellet with mBP centrifugal washing medium, use 190 µL without indicator mBP medium suspension, as observed before and cultured. According to 1:1 proportion (CFU) the above

bacterial culture was mixed pairwise, taking 1 mL centrifugal, the precipitate was diluted with bacteria to pasteurized milk 1×10^2, 1×10^3, 1×10^4, 1×10^5 with 1×10^6 CFU mL^{-1}, taking 1 mL centrifugal, bacterial pellet with 190 µL without indicator mBP medium suspension, as observed before and cultured .

Clinical observation on the cultivation of milk samples: The blood agar plate culture results, select *aureus* positive milk samples 60 and negative parts of milk samples 40, taking 1 mL with an equal amount of PBS mix at the 12000 rpm min^{-1} centrifugal 10 min bacterial pellet with PBS washed by centrifugation, use 190 µL without indicator mBP medium suspension, as observed before and cultured. Another sample of each milk 100 µL, coating Baird-Parker agar plates 37°C to cultivate 24 h observations.

Statistical analysis: Chi-square test was performed using SPSS Software version 22 (IBM SPSS Statistics for Windows, Armonk, NY, USA: IBM Corp.) to compare the *Staphylococcus aureus* between the studied of positive and negative samples of milk. For the test, $p<0.05$ was considered statistically significant.

RESULTS

Determination of the bacterial concentration: To facilitate quantitative test bacteria, first measured using a spectrophotometer *Staphylococcus aureus* mastitis, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus uberis* and *E. coli* cultures D600 nm value and take quantitative dilution broth agar plates coated, after culturing overnight colony counts. The results showed that 1 D600 nm value *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *uberis, E. coli* cultures were 4.7×10^8, 4.7×10^8, 1.19×10^8, 1.28×10^8 with 7.5×10^8 CFU mL^{-1}.

Resazurin selective cultivation of microplate properties: Different concentrations of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylose*, *Staphylococcus aureus*, *Staphylococcus simulans*, hemolysis *Staphylococcus*, *dysgalactiae, Streptococcus agalactiae*, *Streptococcus uberis* and *Escherichia coli* were cultured in micro-plates resazurin in 12 h, the medium color is changed from blue to purple (pink) red determine whether bacterial growth. The medium color greater than or equal ++ Judged as positive for bacterial growth. The results show that when the concentration of bacteria 1×10^2 CFU mL^{-1} time, *Staphylococcus aureus*, *Staphylococcus xylose*, Chromogenic mimic the growth of *Staphylococcus aureus* and positive, other growth-negative bacteria; while when the concentration of bacteria $\geq1\times10^3$ CFU mL^{-1} time, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylose*, *Staphylococcus aureus*, *Staphylococcus simulans*, Hemolytic staphylococci positive growth, *dysgalactiae, Streptococcus agalactiae, uberis* and *E. coli* were negative for growth[12-14] as shown in the Table 1.

Resazurin microplate of *Staphylococcus* growth: Different concentrations of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylose*, *Staphylococcus aureus*, *Staphylococcus simulans* and *Staphylococcus haemolyticus* cultured in micro-plate's resazurin 12 h at intervals of 1 h observations. The results showed that when the concentration of bacteria 1×10^2 CFU mL^{-1} time, *Staphylococcus aureus*, *Staphylococcus xylose*, *Staphylococcus aureus* and *Staphylococcus simulans* cultures 10 h is the positive growth, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* culture 12 h growth is still negative, while when the concentration of bacteria is 1×10^3 CFU mL^{-1} time, *Staphylococcus aureus*, *Staphylococcus xylose*, *Staphylococcus aureus* and *Staphylococcus simulans* cultures 10 h growth is positive, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* culture 12 h growth is positive. Similarly, when the concentration of bacteria is 1×10^4 CFU mL^{-1} time, the *Staphylococcus aureus*, *Staphylococcus xylose*, *Staphylococcus aureus* and *Staphylococcus simulans* cultures 6 h growth is positive, *Staphylococcus epidermidis* and *Staphylococcus*

Table 1: Growth characteristics of different bacteria on resazurin trace cultural

	Growth characteristics of different bacteria at different concentration			
Bacteria	1×10^2	1×10^3	1×10^4	1×10^5
S. aureus	++	+++	++++	++++
S. epidermis	+	++	+++	++++
S. xylosus	++	+++	++++	++++
S. chromogens	++	+++	++++	++++
S. simulans	+	++	+++	++++
S. haemolyticus	+	++	+++	++++
S. agalactiae	-	-	-	-
S. dysgalactiae	-	-	-	-
S. uberis	-	-	-	-
E. coli	-	-	-	-

The medium does not change color (no growth of bacteria), +: Mild color medium (25% bacterial growth), ++: Medium to moderate discoloration (50% bacterial growth), +++: Medium purple-red (75% bacterial growth) and ++++: Medium pink (100% bacterial growth)

Table 2: Dynamic growth of staphylococci on resazurin microplates

Bacteria	Concentration (CFU mL^{-1})	Cultivation results for different cultivation time									
		3	4	5	6	7	8	9	10	11	12
S. aureus	1×10^2	-	-	-	-	-	-	+	++	++	+++
	1×10^3	-	-	-	-	-	+	+	++	++	+++
	1×10^4	-	-	+	++	++	+++	+++	++++	++++	++++
	1×10^5	-	-	+	++	+++	++++	++++	++++	++++	+++
S. epidermis	1×10^2	-	-	-	-	-	-	+	++	++	+++
	1×10^3	-	-	-	-	-	+	+	++	++	+++
	1×10^4	-	-	+	++	++	+++	+++	++++	++++	++++
	1×10^5	-	-	+	++	+++	++++	++++	++++	++++	++++
S. xylosus	1×10^2	-	-	-	-	-	-	+	++	++	+++
	1×10^3	-	-	-	-	-	+	+	++	++	+++
	1×10^4	-	-	+	++	++	+++	+++	++++	++++	++++
	1×10^5	-	-	+	++	+++	++++	++++	++++	++++	++++
S. chromogens	1×10^2	-	-	-	-	-	-	+	++	++	+++
	1×10^3	-	-	-	-	-	+	+	++	++	+++
	1×10^4	-	-	+	++	++	+++	+++	++++	++++	++++
	1×10^5	-	-	+	++	+++	++++	++++	++++	++++	++++
S. simulans	1×10^2	-	-	-	-	-	-	+	++	++	+++
	1×10^3	-	-	-	-	-	+	+	++	++	+++
	1×10^4	-	-	+	++	++	+++	+++	++++	++++	++++
	1×10^5	-	-	+	++	+++	++++	++++	++++	++++	++++
S. haemolyticus	1×10^2	-	-	-	-	-	-	-	-	-	+
	1×10^3	-	-	-	-	-	-	-	-	+	++
	1×10^4	-	-	-	-	-	+	+	++	+++	+++
	1×10^5	-	-	+	++	++	+++	+++	+++	+++	+++

Medium does not change color (no growth of bacteria), +: Mild color medium (25% bacterial growth), ++: Medium to moderate discoloration (50% bacterial growth), +++: Medium purple-red (75% bacterial growth) and ++++: Medium pink (100% bacterial growth)

haemolyticus culture 10 h growth is positive, while when the concentration of bacteria is1×10^5 CFU mL^{-1} time, while 6 kinds of *S. aureus* culture for 6 h were positive growth[14-16] as shown in the Table 2.

Simulated milk samples of resazurin trace Board training: In view of *Staphylococcus aureus* and *Staphylococcus xylose*, production growth *Staphylococcus aureus* and the same dynamic imitation, *S. epidermidis* staphylococci growth dynamics same hemolysis, select *Staphylococcus aureus* and *S. epidermidis* strains tested as representatives of *Staphylococcus aureus* mastitis Cows[17,18]. Pasteurized milk with *Staphylococcus aureus*, *Staphylococcus epidermidis*, *dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus* and big breasts *Enterobacter* pure culture was diluted different fold, taking 1 mL centrifugal, precipitated bacterial culture micro-plate resazurin results showed, when the concentration of bacteria 1×10^2 CFU mL^{-1} time, to cultivate 12 h no growth of *Staphylococcus aureus*, while when the concentration of bacteria 1×10^3 CFU mL^{-1} time, to cultivate 12 h visible growth of *S. aureus*, no growth of *Staphylococcus epidermidis*. Similarly, when the concentration of bacteria 1×10^4 CFU mL^{-1} time, to cultivate 10 h visible growth of *S. aureus*, to cultivate 11 h visible growth of *Staphylococcus epidermidis*; when the concentration of bacteria is 1×10^5 CFU mL^{-1} time, to cultivate 8 h visible growth of *S. aureus*, to cultivate 9 h visible growth of *Staphylococcus epidermidis*. However, when the concentration of bacteria is 1×10^6 CFU mL^{-1} time, to cultivate 8 h visible *Staphylococcus aureus* and *Staphylococcus epidermidis* growth (Table 3)[16,17]. Bacterial concentrations in the range of the above, to cultivate 12 h there were no *dysgalactiae*, *Streptococcus agalactiae* and *uberis* and *E. coli* growth. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis* and *E. coli* culture for combinations of two pure, with pasteurized milk was diluted to 1×10^5 CFU mL^{-1}, taking 1 mL centrifugal, precipitated bacterial culture micro-plate resazurin results showed, *Staphylococcus aureus* and a combination of non-culture 8~9h visible bacterial growth, *Staphylococcus epidermidis* in combination with a non-culture 9-10 h visible bacterial growth, culture of *E. coli* in combination with *Streptococcus* 12 h Yet to see the growth of bacteria (Table 3)[18]. Another *Staphylococcus aureus* and *Staphylococcus epidermidis* each 8 repetitive strain test, the results obtained are consistent[6].

Resazurin micro-plate, rapid diagnostic test applications: Select aureus positive milk samples 60 and negative parts of milk samples 40, take 1 mL centrifugal, the precipitated

Table 3: The cultivation results of milk samples mimicking singular bacterial infections

		Cultivation results for different cultivation time									
Bacteria	Concentration (CFU mL^{-1})	3	4	5	6	7	8	9	10	11	12
S. aureus	1×10^2	-	-	-	-	-	-	-	-	-	+
	1×10^3	-	-	-	-	-	-	-	-	+	++
	1×10^4	-	-	-	-	-	-	+	++	+++	++++
	1×10^5	-	-	-	-	+	++	+++	++++	++++	++++
	1×10^6	-	-	-	-	++	++	+++	++++	++++	++++
S. epidermis	1×10^2	-	-	-	-	-	-	-	-	-	-
	1×10^3	-	-	-	-	-	-	-	-	+	++
	1×10^4	-	-	-	-	-	-	+	++	+++	++++
	1×10^5	-	-	-	-	+	++	+++	++++	++++	++++
	1×10^6	-	-	-	-	++	+++	+++	++++	++++	++++

	Cultivation results for different cultivation time									
Bacterial combinations	3	4	5	6	7	8	9	10	11	12
S. aureus+E. coli	-	-	-	-	+	++	+++	+++	+++	+++
S. aureus+S. dsgalactiae	-	-	-	-	-	+	++	+++	+++	+++
S. aureus+S. agalactiae	-	-	-	-	+	++	+++	+++	+++	+++
S. aureus+S. ueris	-	-	-	-	+	++	+++	+++	+++	+++
S. epidermis+E. coli	-	-	-	-	-	+	++	+++	+++	+++
S. epidermis+S. dysgalactiae	-	-	-	-	-	-	+	++	+++	+++
S. epidermis+S. agalactiae	-	-	-	-	-	-	+	++	+++	+++
S. epidermis+S. uberis	-	-	-	-	-	-	+	++	+++	+++
E. coli+S. dysgalactiae	-	-	-	-	-	-	-	-	-	-
E. coli+S. agalactiae	-	-	-	-	-	-	-	-	-	-
E. coli+S. uberis	-	-	-	-	-	-	-	-	-	-

Medium does not change color (no growth of bacteria), +: Mild color medium (25% bacterial growth), ++: Medium to moderate discoloration (50% bacterial growth), +++: Medium purple-red (75% bacterial growth) and ++++: Medium pink (100% bacterial growth)

Table 4: Cultivation results of clinical milk samples

	Staphylococcus-positive (n = 60)		*Staphylococcus*-negative (n = 40)	
Methods	Sample counts (+)	Agreement (%)	Sample counts (-)	Agreement (%)
Resazurin micro-plate	59	98.3	38	95.0
BP Baird-Parker plate	59	98.3	40	100

bacterial culture in micro-plate's resazurin 12 h, milk samples from each 100 µL inoculation Baird-Parker agar plates, to cultivate 24 h as comparison. The results showed that in 60 parts of the milk samples positive staphylococci, resazurin micro-plate and Baird-Parker agar plates were culture positive 59 parts; in 40 parts by *Staphylococcus* negative milk samples, resazurin microtiter plate culture negative for 38 parts, Baird-Parker agar plate culture negative for 40 parts as shown in the Table 4 by Qiu *et al.*[7] and McNicholl *et al.*[8].

DISCUSSION

The mBP medium can also be used for rapid diagnosis of coagulase-negative staphylococci. In this study, mBP added to the medium indicator resazurin sodium, the results showed that only the medium color change may be displayed growth of *S. aureus* and can display *Staphylococcus epidermidis*, *Staphylococcus xylose*, *Staphylococcus aureus*, *Staphylococcus simulans* and *Staphylococcus* hemolysis growth of coagulase-negative staphylococci. In mBP indicator added to the medium resazurin, theoretically will not change mBP culture medium characteristics selected, this was further confirmed in this study. All of the experimental results of the present study have presented clear agreement with the previous study by Xu *et al.*[18], Deurenberg *et al.*[19] and Vanderhaeghen *et al.*[20] who reported that addition of medium resazurin does not change the color of the mBP culture medium. However, resazurin is a redox indicator nonspecific, as long as there exists a number of live bacteria that can cause color changes in the medium[14,15].

This study used two strategies to solve this problem: First, select the appropriate concentration of resazurin by preliminary tests (0.4 g L^{-1}), to minimize non-specific color of resazurin; second, the bacteria culture after the inoculation 2 h coupled with resazurin indicator, for non-staphylococcal growth medium suppression components fully play its role. Selection culture with different bacteria results showed, that

resazurin can display not only the micro-plate 6 dairy cow mastitis *Staphylococcus* major growth, medium discoloration time and concentration and incubation time with the *Staphylococcus* have obvious relevance. In the previous study results are in contradictory finding with our present study results[20]. The concentrations of up to 1×10^6 CFU mL^{-1} the cow mastitis streptococci and *E. coli* culture 12 h they showed no growth, resazurin microtiter plate showed a strong selective culture characteristics *aureus*. *Staphylococcus aureus* is widely present in the body surface and dairy cattle breeding environment, therefore *aureus* culture-positive does not mean milk cow mammary gland has staph infection, this domestic research reports often overlooked[11,12]. According to reports, need milk *S. aureus* concentration $\geq$50 CFU mL^{-1} in order to explain cow mammary *Staphylococcus aureus* infection, other coagulation-negative staphylococci bacteria number concentration and the like $\geq$250 CFU mL^{-1} sentenced to breast corresponding bacterial infection[4]. In the previous study reported that coagulation-negative staphylococci concentration ration $\geq$250 CFU mL^{-1} penalized to breast corresponding bacterial infection[21-23].

Other relevant results of this study and our study showed that the concentration of bacteria in milk staphylococcal mastitis in more than 1×10^3 CFU mL^{-1} or above. Krziwanek *et al.*[24] and Garcia-Alvarez *et al.*[25] reported that the time and concentration ration of the staphylococci mastitis was more than 2×10^3 CFU mL^{-1} and are clearly agreement with the finding results. In order to accurately diagnose whether there is bovine mammary staph infection, in this study first with pasteurized milk will be different dilutions of different bacterial, milk samples were pelleted by centrifugation analog whichever bacterial infection as breast, *Staphylococcus* concentration $\geq1\times10^3$ CFU mL^{-1} as a criterion of breast staphylococcal infections. The results show that the *Staphylococcus aureus* and *Staphylococcus epidermidis* infections analog samples were incubated 12 h can get diagnostic results[16,18]. All of these studies provided a clear agreement with the present work[24,26,27]. In view of *Staphylococcus aureus* and *Staphylococcus xylose*, *Staphylococcus aureus*, dynamic growth of *Staphylococcus* imitation close, growth *Staphylococcus epidermidis* and *Staphylococcus* dynamic close hemolysis, therefore culture 12 h the basic observations the main dairy cow mastitis can diagnose staph infection. With increasing concentration of staphylococci, medium coloration time is gradually shortened, when the concentration of *Staphylococcus* 1×10^6 CFU mL^{-1} time, to cultivate 8 h can get diagnostic results, tip medium according to the color intensity of infection time preliminary judgment mammary *Staphylococcus*. According to the findings by Kenar *et al.*[28] the results are disagreement with the present study results.

On the basis of respectively, with positive and negative staphylococci milk samples tested, the results show that resazurin micro-titer plate rapid diagnosis of staphylococcal positive milk samples in line with rates up 98.3% (n = 60), diagnostic negative staphylococci in line with the milk sample rates up 95.0%, versus Baird-Parker agar plate culture results are very close, tip the established resazurin micro-plate method can directly replace conventional methods for rapid diagnosis of cow milk staphylococcal mastitis comp[1,9]. Seideman *et al.*[14], Xu *et al.*[18] and Loncaric *et al.*[22] reported that resazurin microtiter plate rapid diagnosis of staphylococcal positive milk samples in line with rates up 96%, which are in contradictory with our present results.

CONCLUSION

From the experimental results of the study, it is concluded that the pure bacterial culture plate showed that the resazurin microplate indicated the growth of *S. aureus* i.e., *S. epidermidis, S. chromogens, S. haemolyticus, S. simulans* and *S. xylosus*. Further from the investigated results concluded that the color changing in the medium was due to the staphylococcal concentration and time incubation. Furthermore, from the results of the study, it is concluded that mastitis milk samples showed the diagnosis rate of *S. aureus* up to 98.3 and 95.0% for positive and negative milk culture plate respectively. Finally, from the results, it was recommended that this resazurin micro-plate can replace the conventional method for rapid diagnosis of *Staphylococcus* milk samples.

SIGNIFICANCE STATEMENTS

This study discovers the estimation of the contaminant-degrading microorganisms by microplate method that can be beneficial for the diagnosis of bacteria in milk. This study will help the researcher to uncover the critical areas of resazurin micro-plate assay that many researchers were not able to explore. Thus, a new theory on these arrived that, the color changing was observed due to the staphylococcal concentration and time incubation interval.

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