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Evaluation of bacterial indicators for antibiotic test assay using resazurin-based reactions in raw milk

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Abstract

Dairy cows often develop several health issues; including bovine mastitis, diarrhea, and pulmonary diseases. Nowadays, antibiotic use is still a common practice among dairy farmers, for both therapeutic and prophylactic purposes, leading to the occurrence of antibiotic residual being left in raw milk. Hence, the detection of antibiotics in raw milk is therefore necessary. Currently, the detection kits use usually apply an assay based on bacterial growth inhibition, and most of these are a single-test assay requiring additional instruments for testing in the laboratory. This study aimed to develop a simple detection method for antibiotics in raw milk using a resazurin-based assay. Six bacterial indicators; including, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella* spp., *Salmonella* Enteritidis, *Staphylococcus aureus*, and *Bacillus* spp. were preliminary screened for their antibiotic-resistant properties against ampicillin and ceftriaxone via minimum inhibitory concentration test. Due to the broad susceptibility to both antibiotics, only *S. aureus* was selected as a bacterial indicator for further study. The limit of detection (LOD) of antibiotic residues was investigated through color changes in the resazurin reaction, and a measurement of optical density at the wavelengths of 600 and 570 nm. The LOD of ampicillin and ceftriaxone residues were 1.0 and 2.0 μg/mL, respectively. Overall, a developed resazurin-based assay could be used to determine residues between 1 to 256 μg/mL for ampicillin and 2 to 256 μg/mL for ceftriaxone, which also allows for the detection of antibiotics in raw milk at room temperature; rather than at higher temperatures than the other test kits currently available.

Keywords: Antibiotics, Bovine mastitis, Raw milk, Resazurin, Microbial detection

1. Introduction

Raw milk contains important sources of nutrition for human health; including, protein, essential amino acids, fat, high water activity and neutral pH. However, raw milk can also support the growth of a variety of microorganisms, including those from the environment and particular pathogens; such as, *Klebsiella*, *Enterobacter*, *Shigella*, *Staphylococcus aureus* (*S. aureus*), and *Escherichia coli* (*E. coli*) [1]. The presence of pathogens in raw milk has been linked to a history of unhealthy animals and infections with pathogens; such as, *S. aureus*, *Streptococcus agalactiae*, and *Pseudomonas aeruginosa* (*P. aeruginosa*). These pathogens are the major causes of mastitis, diarrhea, and pulmonary diseases, resulting in lower milk yields, lower milk quality and price, and the farmers' inability to deliver raw milk [2]. As a result, antibiotics are still widely used for both therapeutic and preventive purposes.

Currently, beta-lactam antibiotics that include ampicillin, penicillin, cephalosporins and ceftriaxone are among the most often used antimicrobial drugs in veterinary medicine to treat microbial infections and illnesses [3-5].

This class of antibiotics inhibits the bacterial cell wall production, resulting in cell lysis and death [6-8]. The presence of antibiotic residue in raw milk, from excessive use of antibiotics, has the effect of low quality, raw milk being produced, which further leads to a reduction in the price of raw milk. This can also lead to a negative impact on the consumers' health; as this could cause carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, bone marrow toxicity, allergies and the occurrence of multidrug-resistant bacteria [9-11]. Therefore, it is crucial for raw milk collection centers to perform antibiotic screening in raw milk once they receive it. Most current, available methods for antibiotic screening in raw milk requires additional instruments in the laboratory, and most methods are a single-test assay; thus, delaying a generation of test results.

Hence, this research was a preliminary study aimed at developing a low-cost detection kit for antibiotics (ampicillin and ceftriaxone) in raw milk, based on the microbiological inhibition method. Six bacterial indicators including *E. coli*, *P. aeruginosa*, *Shigella* spp., *S. enterica* serovar Enteritidis (*S.* Enteritidis), *S. aureus*, and *Bacillus* spp. were preliminary screened for their antibiotic-resistant properties against ampicillin and ceftriaxone via minimum inhibitory concentration (MIC) test. The Clinical and Laboratory Standards Institute (CLSI), whose interpretation cutoffs for antibiotics are based on MIC distributions, is one of the most widely used guidelines in the world [12,13]. The MIC value was combined with the colorimetric method, this being the resazurin-based assay, for further, direct detection of active bacterial cells. Resazurin (blue) is reduced to resorufin (pink), resulting in easy detection of resazurin and resorufin by its maximum absorbance at the wavelengths (λ) of 600 and 570 nm, respectively. This principal and high-throughput of a resazurin-based assay has been widely used in research for antibacterial activity of antibiotic screening assays [14]. Therefore, this technique is useful for our study to develop a simple method for detecting antibiotics in raw milk, using a resazurin-based assay that can operate at room temperature. The assay will be modified further to allow for the testing of multiple samples in one run, or for high throughput antibiotic screening.

2. Materials and methods

2.1 Preparation of raw milk samples

Raw cow milk was purchased from the dairy center at Kasetsart University, Bangkok, Thailand. The raw cow milk was stored at 4ºC overnight (< 12 h), until investigations were carried out. Resazurin tablets for milk testing (VWR Chemicals, United Kingdom) were prepared by dissolving one tablet in 50 mL of sterile glass-distilled water to obtain 52% of stock concentration for further preparation of a 10% (v/v) concentration.

2.2 Preparation of bacterial cultures

Bacterial strains representing environmentally relevant species in raw milk; including, *E. coli*, *P. aeruginosa*, *Shigella* spp. *S.* Enteritidis, *S. aureus*, and *Bacillus* spp. were included in this study. All strains were kindly received from the Bacteriophage and Food Safety Laboratory, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Thailand. These were cultured in tryptic soy broth (TSB; Himedia) at 37°C overnight, and then re-streaked on tryptic soy agar (TSA; Himedia) to obtain a single colony. A single colony of a given bacteria was used to prepare an overnight culture in 5.0 mL of Muller Hinton Broth (MHB; Himedia).

2.3 Determination of bacterial sensitivity and MIC of ampicillin and ceftriaxone

Ampicillin and ceftriaxone were tested for their MIC on each bacterial indicator. Ampicillin sodium salt (AMRESCO, USA) and ceftriaxone (Biochem Pharmaceutical Industries Ltd, India) were prepared and diluted to 1:2 folds, following the manufacturer's instructions, to obtain the concentrations of 0.5 to 256 µg/mL. The broth microdilution method was performed by following the standard protocol of CLSI [12]. Briefly, 50 µL of a given bacterial culture containing 10^5 to 10^6 Colony forming unit (CFU)/mL was mixed with 150 µL of each antibiotic concentration in each well of a 96-well plate. Each reaction with a total volume of 200 µL was incubated at 37°C for 24 h. Monitoring of the turbidity bacterial culture in each reaction was performed by optical density (OD) measurement at the wavelength of 600 nm (as indication of growth), using the FLUOstar Omega microplate reader (BMG LABTECH, Germany). The MIC, which is defined as the lowest concentration at which there is no visible turbidity, was determined for each replicate. After examination for turbidity, each treatment was confirmed for bacterial growth by the streak method on TSA plates, followed by incubation at 37ºC for 24 h.

2.4 Determination of the limit of detection (LOD) for testing ampicillin and ceftriaxone using a resazurin-based assay in raw milk

Raw milk samples, or cell suspension of the bacterial indicator was confirmed for the initial bacterial count on TSA. Raw milk (65 µL) was mixed with 65 µL of each concentration of ampicillin or ceftriaxone; 50 µL of a bacterial suspension containing 10^5 to 10^6 CFU/mL, and 20 µL of 10% (v/v) concentration of resazurin in each well of a 96-well plate. Each reaction, with a total volume of 200 µL, was incubated at 37°C for 3 h. This was followed by the monitoring of changes in color and absorbance of each reaction, using the FLUOstar Omega microplate reader (BMG LABTECH, Germany), for the detection of resazurin and resorufin; at the wavelengths of 600 and 570 nm, respectively. The percentage of relative absorbance change of resazurin and resorufin was calculated according to the following equation (1).

$$Relative\ absorbance\ change\ (\%) = \frac{OD_{final} - OD_{inital}}{OD_{inital}} \times 100 \qquad (1)$$

3. Results

3.1 Selection of a suitable bacterial indicator for testing ampicillin and ceftriaxone

Six bacterial indicators were screened for their susceptibility to ampicillin and ceftriaxone, and for various ranges of the MIC values; as shown in Table 1. For ampicillin, three indicators; including, *E. coli*, *P. aeruginosa* and *Shigella* spp. were resistant as in these are the MIC values of 64, 256 and 32 µg/mL, respectively. Another three indicators; including, *S.* Enteritidis, *S. aureus* and *Bacillus* spp. were susceptible, while presenting with a MIC of 1, 1 and 2 µg/mL. For ceftriaxone, *Shigella* spp. was resistant, while presenting with a MIC value of 32 µg/mL. *P. aeruginosa* showed intermediate susceptibility, while presenting with a MIC value of 16 µg/mL. Additionally, four indicators; including, *E. coli*, *S.* Enteritidis, *S. aureus* and *Bacillus* spp. were susceptible, while presenting with a MIC of 1, 2, 2 and 2 µg/mL, respectively.

For this study, only *S. aureus* was selected as the most suitable indicator for further analysis, due to its susceptibility to both antibiotics tested, while also presenting as a major infectious agent of bovine mastitis.

Table 1 Susceptibility to ampicillin and ceftriaxone in addition to the MIC values of bacterial indicators.

Bacterial indicator	MIC in µg/mL (Susceptibility[a])	
	Ampicillin	Ceftriaxone
Escherichia coli	64 (R)	1 (S)
Pseudomonas aeruginosa	256 (R)	16 (I)
Shigella spp.	32 (R)	32 (R)
Salmonella Enteritidis	1 (S)	2 (S)
Staphylococcus aureus	1 (S)	2 (S)
Bacillus spp.	2 (S)	2 (S)

[a]Interpretation for antibiotic susceptibility based on CLSI guidelines (2019) [15].

3.2 Determination of the LOD for testing ampicillin and ceftriaxone in raw milk using resazurin-based reactions

The LOD results were obtained after incubation at 37±2°C for 3 h. This presented a complete reaction between the suspension of *S. aureus* and resazurin in raw milk. Typically, raw milk containing an initial bacterial count at 3.6×10^4 CFU/mL would change color, from blue (resazurin) to pink (resorufin), in the control (antibiotic at 0 ug/mL). The three, following conditions were observed in this study (Figure 1): (i) the viability of *S. aureus* suspension was confirmed in the reaction without antibiotics (0 ug/mL), and the color would change from blue to either purple or pink; (ii) in the presence of antibiotics, the color would remain blue or purple, indicating that bacterial growth was inhibited; and (iii) in the presence of antibiotics greater than the LOD, this would still observe cell viability; thus, the color would change from blue to either purple or pink. In this study, the presence of ampicillin at 1 µg/mL, the color remained blue or purple, indicating that bacterial growth was inhibited. For ceftriaxone, no color change was observed at 2 to 256 µg/mL; indicating that bacterial growth was inhibited (data not shown). It is recommended that the LOD for testing ceftriaxone is 2 µg/mL.

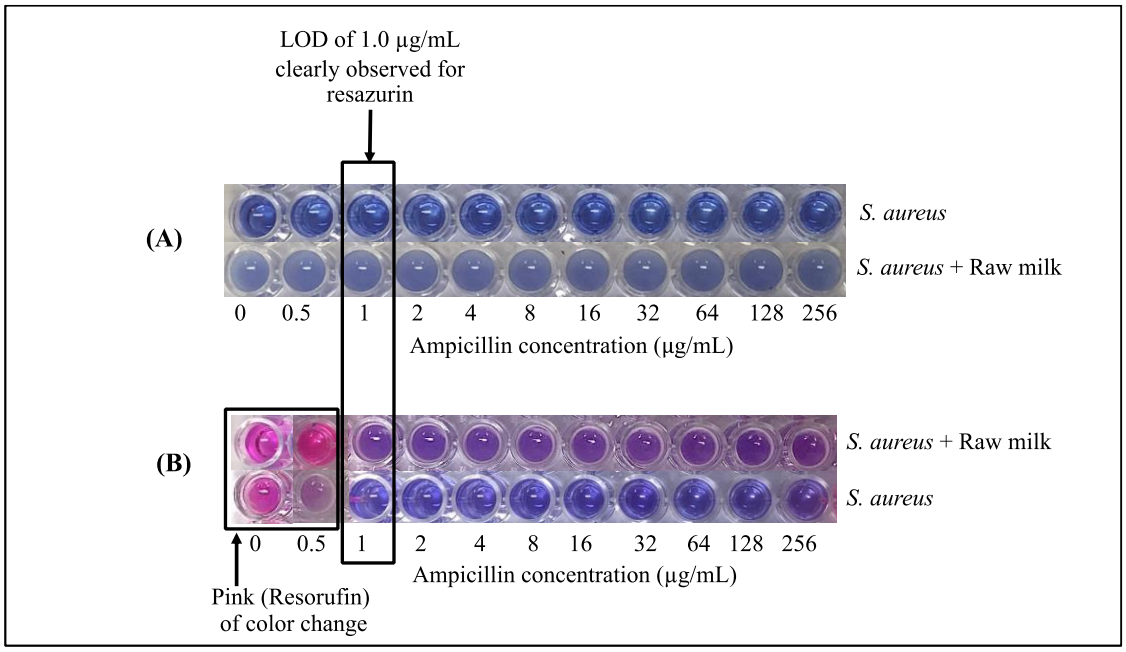

Figure 1 Color changes of the resazurin reaction in the ampicillin testing assay, as observed in cell suspension of *S. aureus*, with an initial concentration of 10^5-10^6 CFU/mL, and *S. aureus* suspension mixed with raw milk before incubation (A) and after incubation (B) at 37±2°C for 3 h.

3.3 The relationship of resazurin and resorufin in the antibiotic testing assay in raw milk

Detection of the color changes were used to monitor the changes in the resazurin reactions by measuring the optical density at the wavelengths of 600 and 570 nm, for the presence of resazurin and resorufin, respectively. In raw milk and *S. aureus* suspension, there was significant difference in the relative absorbance change for both resazurin and resorufin within the reaction. However, there was no significant difference in relative absorbance change for both resazurin and resorufin in the reaction with 0.5 µg/mL of ampicillin as compared to the control ($p > 0.05$) (Figure 2A and 2B). Similarly, there was no significant change for both resazurin and resorufin in the reactions with 0.5 and 1.0 µg/mL of ceftriaxone as compared to the control ($p > 0.05$) (Figure 3A and 3B). These findings suggest that the LOD for antibiotic testing was 1.0 µg/mL for ampicillin and 2.0 µg/mL for ceftriaxone. In raw milk, the results were consistent with those observed in the *S. aureus* suspension.

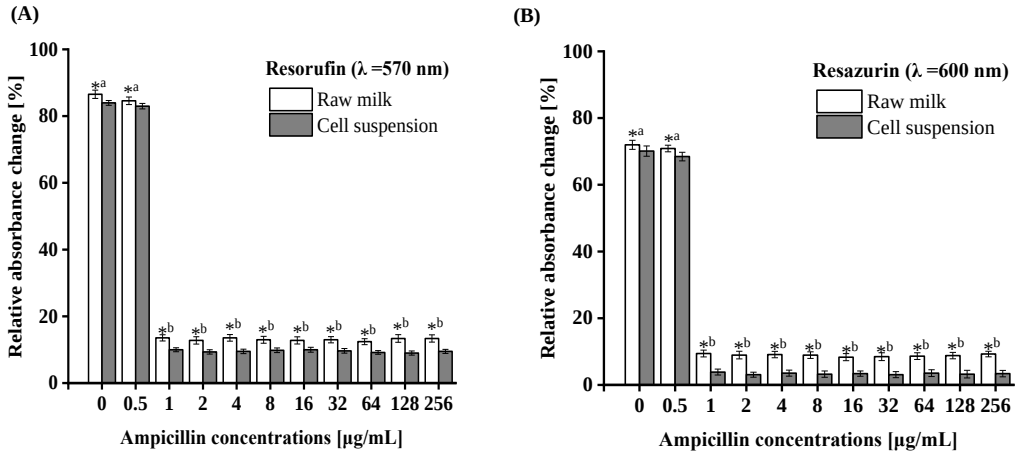

Figure 2 Relative absorbance change (%) of the resazurin reaction in raw milk and *S. aureus* suspension, with different concentrations of ampicillin; (A) resorufin reaction and (B) resazurin reaction. The *asterisk* (*) indicates there were significant differences ($p < 0.05$) between raw milk and cell suspension. The different, lowercase letters for each concentration indicate there were no significant differences ($p > 0.05$) in the change of resazurin or resorufin between a reaction with antibiotics as compared to the control (0 µg/mL).

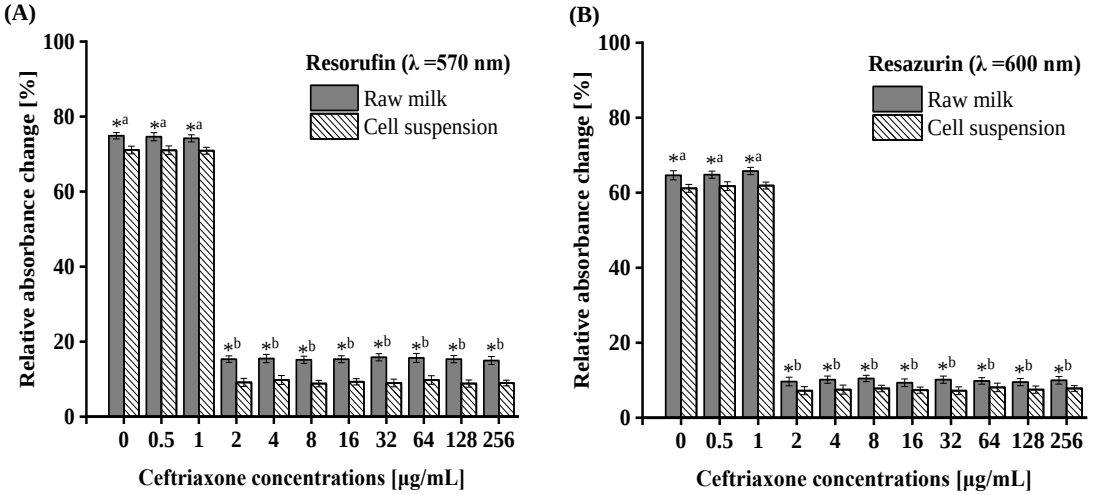

Figure 3 Relative absorbance change (%) of the resazurin reaction in raw milk and *S. aureus* suspension with different concentrations of ceftriaxone; (A) resorufin reaction and (B) resazurin reaction. The *asterisk* (*) indicates there were significant differences ($p < 0.05$) between raw milk and cell suspension. The different lowercase letters for each concentration indicate there were no significant differences ($p > 0.05$) in the change of resazurin or resorufin between a reaction with antibiotics as compared to the control (0 μg/mL).

4 Discussion

In this study, *S. aureus* was selected as a suitable indicator for testing ampicillin and ceftriaxone in raw milk, as this bacterium showed low MIC values for both types of antibiotics. This bacterium is also likely to be the cause of bovine mastitis in dairy cows [16]. *S. aureus* has been reported to carry several antibiotic resistant genes; including those of beta-lactam resistant genes [17,18]. Limya et al. (2020) also reported that *S. aureus* was sensitive to ampicillin, cephalexin and cloxacillin [19]. In addition, *S.* Enteritidis showed to be susceptible to both ampicillin and ceftriaxone [20,21]. Even though *E. coli* presented lower MIC values for ceftriaxone, a previous study reported that *E. coli* showed an increasing resistance rate to antibiotics [22]. The previous studies support the selection of *S. aureus* as an indicator for the antibiotic testing assays in our study, as compared to *E. coli*, *Salmonella* and *Bacillus* spp. From the previous study, *S. aureus*, isolated from various animals, showed an ampicillin MIC value of 2.0 μg/mL [23]; whereas, *S. aureus* isolated from goats with subclinical mastitis showed MIC values of ampicillin and ceftriaxone of 1.0 and 8.0 μg/mL, respectively [24].

The LOD for testing ampicillin and ceftriaxone was determined by using a resazurin-based assay, following the colorimetric technique to monitor the color changes as a result of oxidation-reduction of resazurin (blue) to resorufin (pink) [25]. This technique has been applied to screen the drug susceptibility of *Mycobacterium tuberculosis* [26]. A similar scheme of color change from resazurin reactions in testing drugs, or an antibacterial assay has been reported; which altered resazurin (purple) in the reaction with *S. aureus* into resorufin (pink). This finding indicated that the assay could monitor growth of *S. aureus* in the resazurin reaction at 37°C for 18 to 24 h. [27]. In addition, a 96-well microplate assay-based resazurin reaction was performed to determine the inhibitory effects of amphotericin B and allicin on another microorganism; *Leismania* spp. [28]. Furthermore, the percentage reduction of the resazurin reaction (0-35%) was linearly linked with the reduction of *Trypanosoma cruzi* count (ranged from $2.5x10^4$ to $5 x10^6$ cell/mL) when screening with chemical parasiticides [29].

The percentage of relative absorbance change of the resazurin reactions (resorufin and resazurin) were compared between the reaction in raw milk and *S. aureus* suspension. There was a significant difference for ampicillin and ceftriaxone when tested; as raw milk contains complex components of water (87.0%), fat content (3.7%), protein (3.4%), lactose (4.8%) and other forms of citric acid and minerals [30]. Overall, their components frequently contain intense colorants that interfere with the monochromatic absorbance; especially reactions in these substances: i) raw milk is comprised of various sized fat droplets (an average diameter of 3 μm) resulting in light scatter of an unpredictable manner, and its effects to the refractive index ii) the colloidal properties of the concentrated protein particles (whey and casein) have an average diameter of 120 nm, and can produce scattering effects [31-33]. To overcome these problems, various sample, pretreatment approaches must be used to reduce the above-mentioned problems. The previous studies support the distribution of the complex components in raw milk for dilution of concentrated raw milk before measurement of optical density. This is due to that when raw milk is homogenized, the size of the fat globules decreases from 3 μm to around 1 μm, leading to decreases in the

refractive index as the fat content diminishes [32,34]. Other factors including the temperature of reaction and initial pH of raw milk, causes denaturation of proteins and protein aggregates resulting in interfere light scatter. This research used raw milk with its pH in the range of 6.7-6.8, this property does not affect milk turbidity; as turbidity is generated by the light scattering properties of casein micelles when the pH is up to 11.0 [35,36].

In our study, the color changes from the resazurin reaction observed as well as the percentage relative absorbance change of the resazurin or resorufin in the antibiotic testing assay in raw milk suggested a LOD of 1.0 µg/mL for ampicillin and 2.0 µg/mL for ceftriaxone. This LOD is useful for the dairy industry in the detection of antibiotics (beta-lactam) in raw milk, as typical, raw milk from most farms would show antibiotic residue of 0.004 to 1.0 µg/mL [37]. Overall, the assay in this study could detect antibiotics in the β-lactam group at as low as 1.0 to 2.0 µg/mL, which presents an even lower concentration than that of concern by the US-FDA regulations for raw milk (FDA, 2015) [38].

5 Conclusion

This study evaluated *S. aureus* as a potential bacterial indicator for the antibiotic testing assay in raw milk via a resazurin reactions. A developed assay could detect two antibiotics in the beta-lactam group at as low as 1.0 to 2.0 µg/mL in raw milk, and at a lower temperature (37±2°C) than any test kits currently available. This is a key advantage of this developed method. For further development, this assay will be studied for testing on different groups of antibiotics; such as, sulphonamides, tetracyclines and aminoglycosides, which are also important within the dairy industry. The assay will also be modified to allow for the testing of multiple samples in one run, or for high throughput antibiotic screening.

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