Effect of milk on antibacterial activity of tetracycline against *Escherichia coli* and *Staphylococcus aureus* isolated from bovine mastitis

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Abstract The susceptibility of mastitis-causing *Escherichia coli* and *Staphylococcus aureus* to two commonly used antibiotics, tetracycline and penicillin G, was tested in raw milk and in Muller–Hinton (MH) broth by introducing a pH indicator, bromocresol purple, which was shown to be a simple, sensitive, and rapid method. The minimum inhibitory concentration (MIC) of penicillin G in milk was the same as those in MH broth, whereas the MIC of tetracycline in milk was 4 to 32 times that in MH. An irreversible binding between tetracycline and large molecules of milk, which might be due to a hydrophobic interaction, was demonstrated by a dialysis test, suggesting the observed impairing effect was due to the action of milk on the tetracycline being tested. Further investigation revealed that much of the reduction of tetracycline’s activity in milk was attributable to the milk protein casein, while other heat-sensitive components in milk also play some roles.

Keywords Bovine mastitis · Antibacterial susceptibility · Raw milk · Tetracycline · Penicillin G

Introduction

Bovine mastitis is generally considered to be the most costly disease in dairy industry, causing estimated losses of $100 million per annum in Hokkaido, one of Japan’s main milk production areas (Yamane 2006). The economic impact and the diversified bacterial etiology of this disease have led to the development of various therapeutic strategies. Among these strategies, the identification of pathogens followed by antibiotic treatment is the most frequently used therapy (Grave et al. 1999). Tetracycline is one of the most extensively used antibiotics because of its relative safety, low cost, and broad-spectrum activity against Gram-positive bacteria, Gram-negative bacteria, and atypical organisms such as *Mycoplasma* bacteria (Al-Nazawi 2006). However, therapeutic outcomes generally do not match the results from in vitro susceptibility tests for mastitis causing pathogens. This inefficacy has been considered to be due to factors such as limited knowledge of pharmacokinetic properties of tetracycline in lactating dairy cows, decreased antibiotic activity in the milk phase, and the appearance of resistant bacteria (Constable and Morin 2003).

Currently, the susceptibility test to determine the minimum inhibitory concentration (MIC) of antibiotics against a given bacterial strain is usually carried out by using artificial broth or agar. It is widely accepted that no commonly used artificial medium can simulate the in vivo environment of the bovine udder. Milk is a more appropriate medium for MIC experiments; however, technical issues need to be considered when determining bacterial growth inhibition in milk because it is an emulsion. In the past, direct plate counting was frequently used to determine bacterial growth, but it is labor intensive and time consuming. In view of this, many assays such as those based on reduction of triphenyltetrazolium chloride (Ali-Vehmas et al. 1991) or the use of β-glucuronidase (Fang et al. 1995b) have been developed. Nevertheless, these methods can only be applied to a specific group of pathogens. Traditionally, a pH indicator method is frequently employed to monitor the contamination of phage in the dairy fermentation industry due to its simplicity and ease of
application (Kutter and Sulakvelidze 2005). Based on this concept, a commercially available MASTik® test kit was developed to measure the antibiotic susceptibility of a wide range of mastitis pathogens (George 1993). Unfortunately, limitation of this kit is that it provides no information on the causative agents, and it does not use a standardized inoculum.

Up to now, many researchers have reported reduced activity of tetracycline in milk in vitro compared to its activity in artificial broth media (Owens and Watts 1986; Fang and Vikerpuur 1995a; Fang and Pyörälä 1996) but the reason why tetracycline behaves differently in milk remains unclear. The decreased activity of tetracycline in milk have been suggested to be due to binding to fat, casein micelles, or chelating by bivalent calcium in milk. However, no reliable study attempting to verify these hypotheses has been reported to date.

In this study, we investigated the antibacterial activity of tetracycline against bovine mastitis bacterial isolates in raw milk using a pH indicator, bromocresol purple. This method was designed to overcome the limitations of test kits such as MASTik®. The activity of another widely used antibiotic, penicillin G, was examined in parallel for comparison. Finally, we attempted to clarify the mechanism of the effect of raw milk on the activities of these antibiotics.

**Materials and methods**

**Bacterial isolates**

A total of 12 bovine mastitis-related isolates (six *Staphylococcus aureus* and six *Escherichia coli*) were studied. Mastitic milk samples aseptically taken from affected quarters of cows were collected from Hokkaido. One hundred microliters of appropriately diluted samples were plated onto two types of selective agar media including brain–heart agar and Chromocult® Coliform. Isolates were presumptively identified as *S. aureus* or *E. coli* by phenotypic methods, including morphological observations and catalase, oxidase, and coagulase assays. *E. coli* K12 (w3110) and *S. aureus* ATCC6538 (JCM 2151) were used as reference strains.

**Milk and milk fraction preparation**

Raw bovine milk was aseptically collected from clinically healthy cows with milk somatic cell counts below 70,000 cells/ml in Hokkaido of Japan. For preparation of skim milk, milk samples were centrifuged at 3,000×*g* for 10 min at 4°C, the fat layer was removed with a sterile spatula, and the skim milk was then transferred into another sterile tube. The procedure was repeated until all the fat had been removed.

For preparation of whey, the skim milk was subsequently subjected to centrifugation at 45,000×*g* for 60 min at 4°C. The translucent supernatant was collected and filter-sterilized using 0.22 μm pore size according to the procedures described before (Fang et al. 1995b). For preparation of boiled whey, whey was heated to 100°C for 30 min in a water bath, followed by centrifugation at 8,000×*g* for 10 min at 4°C to remove the precipitate.

Comparison of pH indicator-based technique and the plate counting method for evaluation of bacterial growth in milk

Fifty milliliters raw milk was inoculated with 0.1% v/v of an overnight culture of *E. coli* K12 or *S. aureus* ATCC 6538, respectively. The inoculated samples were incubated at 37°C for a minimum of 25 h. Aliquot samples were taken immediately at different time intervals for parallel pH measurement and plate counting.

**Fluorescence microscopy**

Staining of bacteria by 4’,6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries, Ltd., Japan) was performed as described previously with some modifications (Miyanaga et al. 2007). Briefly, raw milk or Luria–Bertani (LB) was inoculated with 0.1% v/v of an overnight culture of *S. aureus* ATCC6538. The inoculated sample was cultured for 11 h, then mixed with an equal volume of DAPI (final concentration 0.05 mg/ml) and incubated in the dark at room temperature for 5 min. The stained cells were observed through a fluorescent microscope with a cooled charge coupled device camera (DP70, Olympus, Japan) under UV light (330–385 nm). All images were captured under identical conditions (exposure time 10−3 s).

**Broth microdilution assay of antimicrobial susceptibility**

Susceptibility tests were carried out parallelly in Muller–Hinton (MH) broth (Becton Dickinson, Franklin Lakes, NJ, USA), raw milk, skim milk, whey, or boiled whey using the standard broth microdilution method. The antibiotics used in this study were tetracycline-HCl (Wako Pure Chemical Industries, Ltd., Japan) and penicillin G (Nacalai Tesque Inc., Japan); their stock solutions were prepared in saline, filter-sterilized, and stored at −20°C. Prior to the test, the stock solutions were diluted twofold serially in saline to cover the predicted susceptibility range. Twenty-five microliters of serially diluted antibiotics was added to individual wells of a sterile 96-well polyethylene plate (Becton Dickinson, Franklin Lakes, NJ, USA) and mixed with 200 μl of MH broth or 0.005% bromocresol purple (Nacalai Tesque Inc., Japan) supplemented milk, skim, whey, or boiled whey. Next,
single colonies of *E. coli* or *S. aureus* isolates were subcultured in LB and brain–heart medium, respectively (37°C for 12–14 h). Bacterial solution was then adjusted using saline to achieve turbidity equivalent to 0.5 McFarland standard, which is corresponding to approximately 10⁸ CFU/ml bacterial concentration (The National Committee for Clinical Laboratory Standards (NCCLS) 2002). The bacterial suspension was further diluted tenfold by saline to reach a final inoculum concentration of 10⁶ CFU/ml. Twenty-five microliters of the inoculum were added to each well. The negative control consisted of 250 μl of medium alone or containing 0.005% bromocresol purple. The plates were incubated for 18 h at 37°C with ambient air circulation and without shaking. The MIC was determined as the lowest antibiotic concentration at which no cloudiness or no color change was observed in a particular well. All assays were performed in triplicate.

### HPLC analysis

Antibiotic concentrations were analyzed by a reversed phase high-performance liquid chromatography (HPLC) system LC-10AT (Shimadzu Co., Japan). The analytical column was a 4.6×75-mm Zorbax SB-C18 3.5 μm (Agilent Technologies Inc., Santa Clara, CA, USA) with a reliance cartridge guard column.

The determined optimal HPLC-UV/Vis condition for tetracycline analysis required use of a mobile phase consisting of 0.025 M aqueous KH₂PO₄ (pH 3.0): acetonitrile (70:30 v/v) with a flow rate of 1 ml/min. Detector wavelength and column compartment temperature were adjusted to 350 nm and 25°C, respectively.

For analyses of penicillin G, the mobile phase used was 0.025 M aqueous KH₂PO₄ (pH 3.0): acetonitrile (70:30 v/v) with a flow rate of 1 ml/min. Detector wavelength and column compartment temperature were adjusted to 204 nm and 40°C, respectively. The limit of quantitation for each antibiotic was 1 μg/ml and the standard curve was linear within the range from 1 to 10 μg/ml (coefficient of correlation was 0.9989 for tetracycline and 0.9958 for penicillin G).

### Dialysis model

The study was performed in two models using different media: (1) transfer of antibiotic from MH broth to saline (0.85% NaCl solution) and (2) transfer of antibiotic from raw milk to saline. The dialysis system utilized dialysis tube (Spectrum Laboratories Inc., Compton, CA, USA) with a cutoff point at molecular weight of 5,000 Da. All conditions were the same, except that 2 ml of MH broth or raw milk containing 1 mg/ml antibiotic was introduced into each dialysis tube. The tube was placed in a beaker containing 200 ml saline and dialysis was undertaken at room temperature. A sample (0.5 ml) from the saline fluid was taken at appropriate time intervals. The concentration of antibiotic in the samples was determined using the HPLC method described above. Each dialysis experiment was performed three times.

### Results

Relative surface hydrophobicity measurements

The relative surface hydrophobicity, which was defined by the partition between aqueous solution and organic solvent, was investigated. The organic solvents used in this study were hexadecane (Wako Pure Chemical Industries, Ltd., Japan) and chloroform (Nacalai Tesque, Inc., Japan). Briefly, antibiotic solutions (10 μg/ml) were prepared in distilled water. One milliliter organic solvent was added to 1 ml aqueous solution, vortexed for 30 s, and centrifuged for 10 min at 8,000×g. The concentration of antibiotic in the aqueous layer was measured using the HPLC method. The partition value was calculated from the change of antibiotic concentration in the aqueous solution as follows:

\[
\text{Partition(%) = } \frac{C_0 - C_1}{C_0} \times 100
\]

where \(C_0\) = the concentration of antibiotics in aqueous solution before treatment and \(C_1\) = the concentration of antibiotics in aqueous solution after treatment.

### pH indicator-based assay for analysis of bacterial growth in milk

To compare the capacity of the pH indicator-based and the plate counting methods to estimate bacterial growth in milk, both the pH and cell counts were monitored by using two reference strains, *E. coli* K12 and *S. aureus* ATCC6538 (Fig. 1). The progressive reduction of pH in raw milk from 6.81 to 5.69 or 5.61 due to the growth of bacteria was observed over the time and remained within the transition pH range of bromocresol purple. Although the plate counting method showed that the number of *E. coli* K12 increased, *S. aureus* ATCC6538 cell counts decreased before recovering. The behavior of *S. aureus* ATCC6538 in raw milk and LB was examined by fluorescent staining. In the case of raw milk, staphylococcal cells were organized in clumps and tended to adhere to the milk fat globules (Fig. 2b, c), while in LB, clumping was not observed and cells were distributed throughout the sample (Fig. 2a).
Effect of raw milk on the antibacterial activity of tetracycline and penicillin G

Six *S. aureus* and six *E. coli* isolates, both of which are major pathogens associated with mastitis, were obtained from mastitic bovine milk samples. A broth microdilution susceptibility assay was used to compare the activity of tetracycline and penicillin G against the 12 isolates and reference strain of both types in raw milk and ordinary MH broth. Susceptibility was evaluated according to NCCLS guidelines.

Of the 14 strains tested, 13 were sensitive to tetracycline in MH broth. The MIC value of tetracycline was estimated to be $\leq 0.5$ μg/ml for all seven Gram-positive *S. aureus* and $\leq 1$ μg/ml for six out of seven Gram-negative *E. coli*. However, the MIC values of tetracycline on all the test organisms were 4 to 32 times those in MH (Table 1), indicating that milk has an inhibitory effect on the interaction between tetracycline and the organisms tested.

In the case of penicillin G, no significant differences were observed between the MICs measured in MH and those measured in raw milk (Table 1). However, it demonstrated great growth inhibitory effect on only six strains out of the 14 tested. All the *E. coli* isolates exhibited resistance to penicillin G, with MIC $\geq 16$ μg/ml. Furthermore, there was strain-related variation in susceptibility to penicillin G within the group of *S. aureus* isolates. For example, *S. aureus* isolate 26 was shown to be more resistant to penicillin G.

Binding of tetracycline to components of raw milk

The observed inhibitory effect of milk on the antibacterial activity of tetracycline against tested organisms was suspected to be due to the adsorption of tetracycline to milk. To test this hypothesis, a series of experiments were performed. Diffusion of 1 mg/ml tetracycline or penicillin G through a dialysis...
membrane was observed in two different dialysis systems (MH to saline and raw milk to saline), as depicted in Fig. 3. In the MH system, the tetracycline concentration in saline buffer reached 9.5 μg/ml after incubation for 31 h and remained constant thereafter. This value was comparable with that calculated according to noninteracting equilibrium dialysis theory which is over 9.9 μg/ml. However, when dissolved in raw milk, the diffusion rate of tetracycline across the membrane decreased markedly. At the time of 31 h, the tetracycline concentration in the saline buffer was 5.53 μg/ml, representing just 58.2% of the concentration measured in the MH system. Even after 45 h of incubation, at which the equilibrium reached in raw milk system, 71% of the tetracycline contained in milk had diffused across the membrane. A comparison of the two diffusion patterns indicates a rapid and high degree of irreversible binding between tetracycline and components with large molecules (>5,000 Da) in raw milk. At the time of 31 h, the tetracycline concentration in the saline buffer was 5.53 μg/ml, representing just 58.2% of the concentration measured in the MH system. Even after 45 h of incubation, at which the equilibrium reached in raw milk system, 71% of the tetracycline contained in milk had diffused across the membrane. A comparison of the two diffusion patterns indicates a rapid and high degree of irreversible binding between tetracycline and components with large molecules (>5,000 Da) in raw milk. Furthermore, by decreasing the initial concentration of tetracycline in raw milk from 1 to 0.5 mg/ml in dialysis system, the extent of irreversible binding increased from 29.0% to 36.8%, implying that the binding is tetracycline concentration dependent.

On the other hand, the equilibrium dialysis patterns of penicillin G in two systems did not reveal a profound difference, although the diffusion rate in raw milk system was a little slower, which might due to a lesser amount of binding of penicillin G to milk components. However, it was observed that the concentration of penicillin G in the MH system stabilized at 9.9 μg/ml by 25 h, and the same concentration was reached in raw milk system 5 h later, indicating this binding might be reversible even if it did occur.

The physicochemical properties of antibiotics are generally considered the major determinants for the extent of their interactions with other substances. The relative surface hydrophobicity of tetracycline and penicillin G, which is an important physicochemical parameter, was evaluated by examining the ratio of antibiotics absorbed by two organic solvents, hexadecane and chloroform (Table 2). Penicillin G possessed very poor solubility in both organic solvents, while the solubility of tetracycline was 13.3 times or 8.75 times that of penicillin G, indicating that the relative surface hydrophobicity of tetracycline is higher.

Inhibitors to the antibacterial activity of tetracycline in raw milk

To determine which components of milk might be responsible for the irreversible binding and thus inhibit tetracycline bioavailability, a reference E. coli K12 was tested for susceptibility toward tetracycline in skim milk and whey (Table 3). Compared to MH broth, a 16-fold increase in tetracycline concentration was required to eliminate the growth of E. coli K12 in skim milk, which is devoid of fat. The inhibition level exhibited by skim milk was the same as that in raw milk. On the other hand, whey which lacks casein caused less inhibition. The activity of tetracycline in whey increased eight times compared to that in skim or raw milk, and further treatment of the whey by boiling increased bioavailability of tetracycline to levels comparable with those reached in MH broth.

<table>
<thead>
<tr>
<th>Staphylococcus aureus</th>
<th>Penicillin G MIC (μg/ml)</th>
<th>Tetracycline MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH broth</td>
<td>Raw milk</td>
<td>Ratio</td>
</tr>
<tr>
<td>Isolate 2</td>
<td>0.015625</td>
<td>0.015625</td>
</tr>
<tr>
<td>Isolate 3</td>
<td>0.03125</td>
<td>0.03125</td>
</tr>
<tr>
<td>Isolate 19</td>
<td>0.015625</td>
<td>0.015625</td>
</tr>
<tr>
<td>Isolate 20</td>
<td>0.015625</td>
<td>0.015625</td>
</tr>
<tr>
<td>Isolate 21</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Isolate 26</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>ATCC 6538</td>
<td>0.03125</td>
<td>0.03125</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Escherichia coli</th>
<th>Penicillin G MIC (μg/ml)</th>
<th>Tetracycline MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate 1</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Isolate 2</td>
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</tr>
<tr>
<td>Isolate 12</td>
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<td>Isolate 20</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>K12</td>
<td>1,024</td>
<td>1,024</td>
</tr>
</tbody>
</table>

Table 1 Antibacterial activity of penicillin G and tetracycline in MH broth and raw milk

*Ratio=MIC value of each antibiotic in raw milk/MIC value of each antibiotic in MH broth
Discussion

The problem of using antibiotic therapy in milk to treat bovine mastitis has been recognized for some time (Craven 1987). There is a suspicion that susceptibility testing of mastitis pathogens has not been adequately validated for most pathogens and antibiotics. This has led to a search for alternative explanations of failure mechanisms (Sandholm et al. 1990). Our results confirm the problem of artificial susceptibility test in bovine mastitis. In addition, we found direct analysis of bacterial growth in milk by plate counting method is very difficult and inaccurate, because some bacterial species such as *S. aureus* have a tendency to bind to fat globules followed by aggregation in raw milk. The observation in this study confirms results obtained by other groups (O’Flaherty et al. 2005). These characteristics of *S. aureus* might be a virulence determinant in the development of bovine mastitis, especially as a chronic disease. On the contrary, the pH indicator-based method was clearly shown to be simple and sensitive for antibiotic susceptibility testing in milk. Since most mastitis pathogens are lactose-fermentation bacteria, the growth of these bacteria will ferment the lactose in milk to lactic acid, with an accompanying decrease in pH (George 1993). Consequently, a color change from purple to yellow will occur when the

**Table 2** The partition values of penicillin G and tetracycline in two organic solvent/water combinations

<table>
<thead>
<tr>
<th>Partition value</th>
<th>Penicillin G</th>
<th>Tetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecane/H₂O</td>
<td>0.6</td>
<td>8.0</td>
</tr>
<tr>
<td>Chloroform/H₂O</td>
<td>4.0</td>
<td>35.0</td>
</tr>
</tbody>
</table>

**Table 3** Antibacterial activity of tetracycline against *E. coli* K12 in different fractions of raw milk

<table>
<thead>
<tr>
<th>MIC (µg/ml)</th>
<th>MH broth</th>
<th>raw milk</th>
<th>skim</th>
<th>whey</th>
<th>boiled whey</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>16</td>
<td>16</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

![Graph](image-url)
bromocresol purple is present which can be used to determine the MIC. In this study, the pH indicator-based technique showed good agreement with the turbid-metric method in testing bacterial susceptibility in MH (data not show).

Penicillin G and tetracycline are both long-established antibiotic agents and have been widely used in the treatment of bovine mastitis. As long as the intramammary area was infected with penicillin-sensitive pathogens, penicillin G is generally considered to be a first-line antibiotic due to its therapeutic advantages compared with β-lactamase-stable penicillins (Grave et al. 1999). However, the appearance of mastitis-causing staphylococci that are resistant to penicillin G has been reported repeatedly. In some countries, the percentage of resistant isolates reached as much as 50% (Gentilini et al. 2000; Erskine et al. 2002). Our study also detected the existence of penicillin G-resistant staphylococci isolates from Japanese bovine mastitis. This result, together with the fact that penicillin G has a narrow spectrum of activity, might limit its future application. Tetracyclines have emerged as a second choice of antibiotics to beta-lactams for the management of mastitis in dairy herds in certain regions of the world because of their broad spectrum activity and low cost.

However, our results showed that the activity of one of the most widely used antibiotics in the tetracycline group decreased markedly when applied in milk. Compared to MH broth, roughly 4 to 32 times more tetracycline was required to inhibit growth of isolated pathogens in milk. Plausible reasons could be that bacterial growth rate is lowered in milk since fast-growing bacteria have been reported to be more susceptible than the slow growing bacteria (Fang and Pyörälä 1996), or the aggregation of bacteria such as S. aureus in milk as it has been reported that some surface-binding staphylococcal strains are less susceptible to antibacterials than planktonic cultures (Chuard et al. 1993). However, in this experiment milk did not impair the activity of penicillin G, which was tested in parallel. This led to our assumption that the cause of the inhibition might be due to physicochemical and pharmacological properties of tetracycline itself. As demonstrated by our results, tetracycline exhibited a higher hydrophobicity compared to penicillin G and a strong irreversible binding with large molecules which might probably due to the hydrophobic interaction occurred in milk. As a result, the concentration of free tetracycline molecules in milk which had access to bacteria was greatly reduced, necessitating the addition of more tetracycline to achieve the same antibacterial activity in milk as in MH broth.

Currently, the only pharmacokinetics of tetracycline known is based solely on achievable serum and interstitial fluid concentrations in humans after oral administration (Agwu and MacGowan 2006). Neuvonen (1976) showed that tetracyclines can form insoluble complexes with calcium, magnesium, iron, and aluminum, which markedly reduces its absorption in serum. Wellin et al (1977) reported that protein, fat, and carbohydrate in meals reduce the absorption of tetracycline by about 50% in human serum. Since bovine milk is a complex medium, composed of water, proteins (caseins, β-lactoglobulins, α-lactoglobulins, immunoglobulins, bovine serum albumin, and various enzymes), lipids, lactose, metal ions, minerals, vitamins, acid, and gases (O’Flaherty et al. 2005), a parallel mechanism may be at work. By comparing the MIC of tetracycline against E. coli K12 in different fractions prepared from raw milk, we found that fat was not the factor in the impairing effect because the removal of fat alone did not influence the MIC value compared to that in raw milk. Instead, the strongest reduction of MIC value was observed after removing the casein, implying that it might be the major inhibitor in milk. Casein, which account for nearly 80% of milk protein, is organized in micelles that have a very porous and hydrophobic structure. It is possible that tetracycline was tightly entrapped into porous casein micelles through hydrophobic interaction, thus reduces its antibacterial activity. In addition to the potential inhibitory roles of casein toward tetracycline, the remaining twofold difference of MIC in whey and MH broth revealed the presence of other inhibitors. Though the identity of these inhibitors is as yet unknown, their sensitivity to heat indicates that they are probably mediated by a protein or a group of proteins. Further characterization of these inhibitors is desirable.

In conclusion, the present study indicates that the pH indicator-based method is simple and sensitive when applied to the antibiotic susceptibility test. It is clear that tetracycline behaves quite differently in raw milk compared to MH broth. An irreversible binding between tetracycline and milk components which might due to hydrophobic interaction was observed and thus might account for the decreased bioavailability of tetracycline in raw milk. Removing the casein and heat-sensitive substances in raw milk restored the activity of tetracycline to a similar level to MH broth, suggesting a role for these components in decreasing the activity of tetracycline in raw milk. The results obtained in this study implied that current adopted susceptibility test for bovine mastitis was unreliable. Improvements, such as using milk instead of artificial broth, need to be made to guide the correct selection and appropriate dosage of antibiotics.

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