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The Detection Limits of Antimicrobial Agents in Cow's Milk by a Simple Yoghurt Culture Test

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Abstract: The aim of this study was to study performance of Yoghurt Culture Test (YCT) in the detection of antimicrobial residues in milk. For this purpose, the sensitivity of YCT for 15 antibiotics were determined. For each drug, 8 concentrations were tested. The detection limits of YCT at 2.5 h and 4 h incubation were determined (μg kg^{-1}): 15 and 37.5, penicillin G; 4 and 5, ampicillin; 5 and 7.5, amoxycillin; 100 and 200, cephalexin; 80 and 100, cefazoline; 100 and 200, oxytetracycline; 500 and 100, chlortetracycline; 100 and 200, tetracycline; 150 and 200, doxycycline; 200 and 400, sulphadimidine; 500 and 1000, gentamycin; 1000 and 1500, spectinomycin; 400 and 500, erythromycin; 50 and 100, tylosin; 5000 and 10000, chloramphenicol. The YCT detection limits at 2.5 h incubation for ampicillin, cephalexin, tetracycline, oxytetracycline and tylosin are similar to those obtained as Maximum Residue Limit (MRL) according to Regulation 2377/90 EEC as set out by the European Union. In addition the detection limits of YCT for some antibiotics were lower than some of microbial inhibitor test.

Key words: Milk, microbial inhibitor, antibiotic

INTRODUCTION

Antimicrobial agents are administered in therapeutic treatment of cattle and constitute a common cause of the presence of chemotherapeutic drug residues in milk. Mastitis is the most prevalent disease of milk-producing cattle which requires antimicrobial treatment (Suhren, 2002). The presence of certain antimicrobial agent residuals in milk constitutes a potential hazard for the consumer and may cause allergic reactions, interference in the intestinal flora and resistant populations of bacteria in the general population, thereby rendering antibiotic treatment ineffective (Dewdney *et al*., 1991; Currie *et al*., 1998).

From a technological point of view, the residues of antimicrobial agents in milk can produce important losses in fermented products; for example, they inhibit the bacterial fermentation processes involved in the production of some dairy products, such as cheese or yoghurt (Nouws *et al*., 1999; Suhren, 2002).

To ensure human food safety, Maximum Residue Limits (MRLs) have been set out for many antimicrobial agents and different methods of analysis developed for the swift detection of residuals of inhibitors present in milk. For these reasons, several manufacturers have developed commercially available tests both for producers and the dairy industry with the aim of detecting drug residues in milk, among these the microbial inhibitor tests (Reichmuth *et al*., 1997; Suhren, 2002; Suhren and Walte, 2003).

The microbial inhibitor test procedure for detection of drug residues in milk is based on inhibition of spore outgrowth of orgamisms such as *Bacillus stearothermophilus* var. *calidolactis* (Suhren, 2002), *Bacillus cereus* (Suhren and Heeschen, 1993), *Bacillus subtilis* (Aurelli *et al*., 1996), noted visually by interpreting the color change of a pH-indicator present in the test medium. In general, microbiological inhibition tests are used for the screening stage, many of them using *Bacillus stearothermophilus* var. *calidolactis*, such as BRT-AiM®, Delvotest®, CH® -ATK microplate. These screening methods were mainly developed and used with cow milk (Scannella *et al*., 1997). In the case of penicillin-G, most of these procedures can detect between 0.004 and 0.006 IU mL^{-1} of milk, but the responses of the microbial inhibitor tests to other antibiotics or inhibitory residues varies with the compound in question.

The aim of the present research was to study performance of Yoghurt Culture Test (YCT) in the detection of different antimicrobial agents belonging to the most representative groups utilized in veterinary medicine in milk.

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MATERIALS AND METHODS

Experimental design and milk samples: The present study was conducted during June to September 2006 in Mashhad, Northeast of Iran. Fresh and antibiotic-free milk sample was drawn from cow known to be free from any form of medication and sample was transported to the laboratory at 4°C. Appropriate volumes of the milk were then dispensed into two clean and sterile jars and, while one jar was held as a control, the pH of another sample was adjusted to 6.0 using 1 N HCl. The milk in each jar was then warmed in an oven for a period of time known to give a temperature of 45°C and was inoculated with 4% (w/w) of yoghurt culture containing equal mixtures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* that are in regular use in Mashhad dairy plants. The yoghurt culture was prepared by mixing 1 g of well-mixed, fresh yoghurt culture with 99 mL of skim-milk (10% dry milk solids, w/v) that had been heat treated at 95°C for 5 min. After thorough mixing of milk samples with culture, each portion was placed in a water bath at 42°C. Measurements of pH were made immediately using a pH meter and after 1.5, 2.0, 2.5 and 4 h incubation. As an alternative to measurements of acidity, the use of pH indicators was examined in a further trial by adding 0.1 mL of Chlorophenol Red (0.2 in 50% ethanol) to the milk either before incubation.

Antimicrobial solutions and test samples: Antibiotics for preparation of the antimicrobial solutions were stored and handled according to the manufacturer's instructions before being used. Each antimicrobial agent was tested at seven different concentrations and in every case a negative control was included.

Table 1 shows the 15 antimicrobial agents and the concentrations used for the preparations of their solutions. For each concentration, 10 replicates were prepared using antibiotic-free milk samples obtained from individual animals. A total of 80 test samples were analysed for each antimicrobial agent. The pH value of each sample of milk was then adjusted to 6.0 with 1 N HCl and the sample warmed to 45°C. A fresh starter culture was then used to inoculate the contaminated milks, along with a negative control sample (zero antibiotic), at rate of 4% and Chlorphenol Red was added as an indicator. After mixing well, each test sample was placed in a water bath at 42°C. Measurements of pH and consideration of coagulum formation were made at 2.5 and 4.0 h and the colour changes were recorded as well: samples without any change in colour/crud formation were suspected being contaminated with antibiotics and were considered positive.

Table 1: Antibiotic concentrations employed for Yoghurt Culture Test detection limits in cow's milk

Antimicrobials	Concentrations (μg kg^{-1} of milk)
Penicillin G	0, 1.5, 3, 7.5, 12, 15, 37.5, 60
Ampicillin	0, 1, 2, 3, 4, 5, 6, 8
Amoxycillin	0, 2.5, 5, 7.5, 10, 12.5, 15, 20
Cephalexin	0, 20, 40, 60, 80, 100, 200, 300
Cephazolin	0, 60, 80, 100, 120, 150, 200, 400
Sulphadimidin	0, 50, 75, 100, 200, 400, 800, 1000
Chlortetracycline	0, 500, 1000, 1500, 2000, 2500, 3000, 4000
Doxycycline	0, 50, 100, 150, 200, 250, 400, 600
Oxytetracycline	0, 100, 200, 400, 600, 800, 1500, 2000
Tetracycline	0, 100, 200, 400, 600, 800, 1500, 2000
Erythromycin	0, 200, 300, 400, 500, 600, 800, 1000
Tylosin	0, 5, 7.5, 10, 25, 50, 100, 150
Spectinomycin	0, 400, 600, 800, 1000, 1500, 2000, 2800
Gentamycin	0, 500, 1000, 1500, 2000, 2500, 3000, 6000
Chloramphenicol	0, 5000, 10000, 20000, 30000, 40000, 60000, 80000

Statistical analysis: For each concentration, 10 replicates were prepared using antibiotic-free milk samples obtained from individual animals. Data were averaged and analysed statistically using SPSS software (Version 10.0.5). The detection limit of the visual interpretation of the YCT method was estimated as concentrations in which 95% of the results were positive (Molina *et al*., 2003).

RESULTS AND DISCUSSION

The minimum concentrations of the different antibiotics giving positive results in the YCT, i.e., no curd formation or colour shift in the presence of Chlorophenol Red, are shown in Table 2, along with MRL values in accordance with EU regulations.

The detection limit of amoxycillin at 2.5 h incubation was lower than the 7 μg kg^{-1} determined in ewe milk samples by Eclipse 100® (Montero *et al*., 2005) and 6 μg kg^{-1} determined by Suhren and Knappstein (1998). In the case of ampicillin, the level detected in this study was lower than 6 μg kg^{-1} determined by Molina *et al*. (2003). For cephalexin residues, the detection limit was lower than 270 μg kg^{-1} detected by Molina *et al*. (2003) and at 2.5 h incubation was lower than 115 μg kg^{-1} detected by Montero *et al*. (2005). The sensitivity of the test to penicillin was rather disappointing because, at 2.5 h, the YCT appeared less sensitive than any other microbial inhibitor tests, it may be that the *Lac. delbrueckii* ssp. *bulgaricus* component of the culture was not affected immediately by the inhibitor. It has also been reported that mixed cultures of *Str. thermophilus* and *Lac. delbrueckii* ssp. *bulgaricus* are less sensitive than the individual species growing alone and this effect might have altered the results as well (Robinson and Tamime, 2002).

The sensitivity of the YCT to oxytetracycline and tetracycline at 2.5 and 4 h incubation was better than BRT- AiM® (Molina *et al*., 2003), delvotest photometric measurement (Althaus *et al*., 2003) and Eclipse 100®

Table 2: The detection limits of antibiotics in cow's milk by the YCT ($\mu g\ kg^{-1}$)

	Coagulum formation at		
Antibiotics	2.5 h	4 h	MRLs[1]
Penicillin G	15	37.5	4
Ampicillin	4	5.0	4
Amoxycillin	5	7.5	4
Cephalexin	100	2000.0	100
CeFazoline	80	100.0	50
Oxytetracycline	100	200.0	100
Chlortetracycline	500	1000.0	100
Tetracycline	100	200.0	100
Doxycycline	150	200.0	100
Sulphadimidine	200	400.0	100[2]
Gentamycin	500	1000.0	100
Spectinomycin	1000	1500.0	200
Erythromycin	400	500.0	40
Tylosin	50	100.0	50
Chloramphenicol	5000	10000.0	0[3]

[1]: Council Regulation 2377/90 EEC, [2]: Sum of all substances of this group, [3]: Not allowed

(Montero *et al*., 2005). In addition the detection limit of YCT at 2.5 h incubation for these antibiotics can be at similar levels to EU-MRLs.

Other microorganisms could also be assayed in other to be able to detect tetracyclines at levels close to EU-MRLs (100 $\mu g\ kg^{-1}$). Suhren and Heeschen (1993) pointed out that the *Bacillus cereus* var. *mycoides* ATCC 9634 is sensitive to concentrations of less than 100 $\mu g\ kg^{-1}$ of different tetracyclines, while Nouws *et al*. (1998) detected between 10 and 30 $\mu g\ kg^{-1}$ of tetracyclines when using *B. cereus* ATCC 11778.

The sensitivity of the YCT to gentamycin (500 $\mu g\ kg^{-1}$ at 2.5 and 1000 $\mu g\ kg^{-1}$ at 4 h incubation) was better than that reported by Molina *et al*. (2003), Althaus *et al*. (2003) and Montero *et al*. (2005).

In this study the erythromycin detection limit was lower than BRT-AiM® (Molina *et al*., 2003), delvotest phothometric measurement (Althaus *et al*., 2003) and Eclipse 100® (Montero *et al*., 2005). The detection limit for erythromycin is very high compared with the EU-MRLs (40 $\mu g\ kg^{-1}$). The detection limit of tylosin at 2.5 h incubation YCT at similar levels to EU-MRLs. For chloramphenicol residues, the sensitivity of YCT was higher than that reported by Molina *et al*. (2003), Althaus *et al*. (2003) and Montero *et al*. (2005). The EU regulation allow zero tolerance for this antimicrobial agent. For this reason, the use of other methods will be assessed. Kolosova *et al*. (2000) can detect 0.08 $\mu g\ kg^{-1}$ of chloramphenicol when utilizing an indirect competitive ELISA method. Whereas Gaudin and Maris (2001) achieved a detection limit of 0.1 $\mu g\ L^{-1}$ by means of a biosensor immunoassay based on polyclonal antibodies.

The reduced sensitivities of the YCT at 4 h is a reflection of the fact that the concentrations that cause a failure at 2.5 h leave a percentage of cells of one or both organisms unaffected. Consequently, sufficient acidity has been generated at the end of 4 h to form a coagulum and a higher concentration is needed to ensure that too few cells survive to lower the pH to 4.8 or below (Yamani *et al*., 1999).

Nevertheless, the results at 4 h were useful for developing the following protocol:

- Failure to change indicator in 2.5 and 4 h-unacceptable level of inhibitory substances in the milk
- Failure to change indicator in 2.5 h, but change after 4 h-marginal level of inhibitory substances in the milk
- Change of indicator in 2.5 h-inhibitory substances below level of detection

Clearly, the disadvantage of the YCT is that it is not so sensitive to β-lactam antibiotics as some of the commercial kits, but this criticism does not alter the value of the YCT as a practical method of assessment for a dairy.

Overall, it would appear that the YCT employing sensitive strains of *Str. thermophilus* and *Lac. delbrueckii* ssp. *bulgaricus* provides a test for inhibitory substances in milk that is broadly comparable in response to other commercial kits. Obviously, the YCT would not be suitable for use in the laboratory of a Regulatory Authority where the priority is to protect consumers from extremely low levels of β-lactam residues, but use of the YCT could be encouraged in countries where the testing of milk supplies for antibiotics is not mandatory.

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