



Evaluation of some screening tests for detection of β -lactams residues in milk production chain

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ABSTRACT

Despite of useful information on important factors of screening tests for the detection of drug residues in milk, the choice of a suitable test is still difficult. The aim of this study was to evaluate three different tests, including Copan, maximum residue limit (MRL) β -lactam and lactic coagulation test for routine screening of milk containing β -lactam antibiotics. Blank milk samples were added with different concentrations of three β -lactams, including penicillin, amoxicillin, ampicillin, and examined then by all three tests. Both Copan milk and MRL β -lactam tests were easy to use and sensitive to mentioned β -lactams at or below the MRLs established by European Union, but lactic coagulation test was not able to detect them at or even exceeding the safe level of MRLs. Copan test was also easier to interpret and less expensive while the results of MRL β -lactam were obtained in a short time. Depend on purpose, both Copan and MRL β -lactam tests can be applied as a screening test for the detection of β -lactams in raw milk.

1. Introduction

Milk as a nutrient source is an important part of the human diet and has considered essential to optimal human growth and evolution (1). The probable presence of veterinary drug residues in milk, however, can highly treat the consumer health (2-4). In addition, drug residues can interfere with the manufacture of fermented products such as yoghurt and cheese (5,6). To prevent these problems, the dairy industry is interested in screening incoming milk to ensure that levels of antibiotic residues are not exceeding the safe levels of maximum residue limits (MRLs) set by European Union (EU) (7).

According to published reports, β -lactams are the most common antibiotics used in veterinary medicine for the treatment or prevention of bovine mastitis (8,9). Withdrawal time is determined for the excretion of drugs from animal's body; nevertheless, they occasionally remain in milk due to misuse, overdose or failure of adherence to withdrawal time (10).

Various screening tests have been developed to detect antibiotic residues at tolerance levels in milk (7). An ideal method should be fast, inexpensive, simple, and suitable to detect a broad spectrum of antimicrobials and also detection limits must comply with the requirements of MRLs (11). Microbial screening tests are widely used for this purpose. The main drawback is that these tests take several hours to obtain results; consequently, they cannot be used as a rapid test when the results are required within a short time (10,11). In addition, earlier researchers have reported high rates of false positive results when they are evaluated under different circumstances (12,13). In contrast, using immune/receptor tests, results are more specific and can be obtained only in a few minutes. One of the limitations is that they are more expensive than microbial tests (11,13).

This study aimed to evaluate: (I) two different screening tests including a microbial (Copan milk test) and an immune/receptor (MRL β -lactam test) to introduce the most appropriate and practical one for

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monitoring of β -lactams in raw milk. (II) It focused on evaluation of lactic coagulation test for its possible application to detect β -lactams in raw milk as either individual or complementary (concurrent) test with microbial or immune/receptor tests.

2. Materials and methods

2.1 Sample collection

The experiment was conducted in the Food and Drug Control Reference Laboratories Center, Food and Drug Organization. To achieve free-antibiotics blank milk, samples were aseptically collected from clean, healthy, lactating cows that had not been treated with any drugs for 6 months. Blank milk samples were placed in a cold box and transmitted to the laboratory.

2.2 Total bacterial count

As soon as milk samples received to the laboratory, total bacterial count was performed following the standardized method to confirm the quality of milk. Milk samples were serially diluted and pour-plated in a semi-solid nutrient media in duplicate and then incubated at 32° C (90° F) for 72 h to stimulate bacterial growth. Single bacteria (or clusters) grow to become visible colonies that were then counted. All plate counts were expressed as the number of colony forming units per milliliter (CFU/ml) of milk. The rest of the samples were kept at 2-4° C until used for the experiment.

2.3 Sample preparation for β -lactams

β -Lactams including penicillin G, ampicillin and amoxicillin supplied from Daana Pharma Company (Iran) were stored and handled according to the manufacturer's instructions before use. Stock solutions were prepared in distilled water, kept at 2-4° C and used within 4 days. Working solutions were prepared in distilled water as well and used on the same day. Fresh milk free of antimicrobial substances was spiked with working solutions to obtain known concentrations of mentioned β -lactams and finally subjected to different tests in three replicates.

2.4 Screening tests

Copan Milk Test kits (Copan, Italy) and MRL β -lactam kits (Rosa, USA) were supplied by Tehran Pegah Dairy Company. Both tests were performed according to the manufacturer's instructions.

In summary, for the Copan milk test, 100 ml of each concentration was pipetted onto the surface of the test ampoule. The ampoules were incubated at 64° C in a specific water bath flowed by visual reading. A purple color was indicated presence of inhibitory

substances in the milk whereas the color change to yellow demonstrated there are no inhibitory substances in the milk.

MRL β -lactam test was performed by adding 200 ml of each known concentration to the strip, closed and incubated at specific incubator for 8 min. Given the fact that test reader was not available, visual reading was carried out based on color change and movement of control and test line.

2.5 Lactic coagulation test

Yoghurt was prepared according to the instructions for the starter culture mix YC-180 as given by the manufacturer. A quantity of 40 g blank milk was transferred to a series of 250-ml volumetric flask and incubated at 80° C in the water bath. After cooling to optimum temperature of the starter culture (42° C), known concentrations of working solution of all above-mentioned β -lactams were spiked in samples except for one sample with no drug as control test. In the following, 1.5 ml of yoghurt was pipetted into the samples and thoroughly mixed. All volumetric flasks were covered with Nescofilm sealing tissue and placed in an incubator at 45° C for 4 h. The positive and negative results were assessed based on the investigation of the clot density in milk samples. The observation of the clot in milk samples verified presence of β -lactams that are inhibiting growth of the organism's test producing yogurt.

All the analyses were examined in three replicates for all different tests.

3. Results

Aseptically collected milk from lactating cow had a standard plate counts (SPC) less than 300 CFU/ml that confirmed the quality of milk samples for the experiment.

The sensitivity of all three tests was determined by testing different concentrations of each above-mentioned β -lactams in milk samples corresponding to the MRLs established by Council Regulation EEC No. 2377/90. Table 1 shows the results of sensitivity of Copan milk test toward examined β -lactams. Copan milk test detected the concentration of 3 ng/g and more β -lactams in milk. As is shown in Table 1, Copan tests were sensitive to all three β -lactams (limit of detection ≥ 3 ng/g).

Table 2 shows the results of sensitivity of MRLs β -lactam test toward examined β -lactams. MRLs β -lactam test detected the concentration of 3 ng/g and more β -lactams in milk. MRLs β -lactam test was also sensitive to all three β -lactams (penicillin, ampicillin, and amoxicillin).

The result of sensitivity of lactic coagulation test toward examined β -lactams has referred in table 3. According the obtained data, the lactic coagulation

Table 1. Sensitivity of Copan milk test toward examined β -lactams

β -Lactams	Concentration of β -lactams in milk (ng/g)															
	1 ng/g			2 ng/g			3 ng/g			4 ng/g			5 ng/g			
Penicillin	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Ampicillin	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Amoxicillin	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+

Table 2. Sensitivity of maximum residue limits β -lactam test toward examined β -lactams

β -Lactams	Concentration of β -lactams in milk (ng/g)															
	1 ng/g			2 ng/g			3 ng/g			4 ng/g			5 ng/g			
Penicillin	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Ampicillin	-	-	-	-	-	-	+	±	+	+	+	+	+	+	+	+
Amoxicillin	-	-	-	-	-	-	+	+	±	+	+	+	+	+	+	+

Table 3. Sensitivity of lactic coagulation test toward examined β -lactams

β -Lactams	Concentration of β -lactams in milk (ng/g)															
	1	2	3	4	5	10	15	20								
Penicillin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ampicillin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amoxicillin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

test could not detect the concentration of 1-20 ng/g β -lactams in milk or all tested samples containing different concentrations of β -lactams ≤ 20 ng/g coagulated in the specified time.

4. Discussion

In this study, a similar pattern of detection was observed for both Copan and MRL β -lactam test; however, Copan test detected two more samples with a positive result. The limit of detection for both tests was below the MRLs established by EU. These findings are in agreement with literature reported earlier (11,14). The result of lactic coagulation test was entirely different from two other tests. This test was not sensitive to β -lactams either at or exceeding the levels of MRL. All tested samples are containing different concentrations of β -lactams ≤ 20 ng/g coagulated in the specified time.

The results of Copan test was more easily interpreted by examiners than two other tests. The fact that test reader was unavailable for the MRL β -lactam, visual readings of two tests were difficult, and so both test and control lines were not clearly observed. The detection of inhibitory substances in milk using lactic coagulation test was based on the investigation of the clot density in milk. The increase in concentration of β -lactams in milk reduced clot density, nevertheless, interpretation of results was more difficult than Copan and MRL β -lactam test. Similar results were obtained in an earlier study in which clotting density of yoghurt decreased proportional with the increasing doses of antibiotics added to milk (9).

Rapidity, specificity and sensitivity are clearly important characteristics for the choice of a screening test applying in the dairy industry. In developing countries, due lack of safety measures and insufficient monitoring along the production chain, presence of

other inhibitory substances in milk is inevitable. In this case, not only specificity is not suitable for the choice of a screening test, but also a broad spectrum of detection is very useful to monitor more inhibitory substances in milk. Although MRL β -lactam test is fast and sensitive, the high specificity constrains its applications as screening test. This test is based on antibody-antigen reactions that only bond with β -lactams, so the other antibiotics and inhibitory substances cannot be detected. In contrast, Copan milk tests have broader spectrum of detection, which can detect nearly all inhibitory substances in milk. Lactic coagulation test was more demanding, time-consuming, difficult to interpret and not sensitive, as a result, it cannot be applied as a screening test. This test is not common for the screening of the raw milk and mostly applies to determine the suitability of raw milk for the manufacturing of fermented products. To the best of our knowledge, there is no scientific evidence to use this test for the screening of raw or pasteurized milk. Institute of Standards and Industrial Research of Iran has offered it as one of the screening tests for the detection of inhibitory substances in pasteurized milk (15).

5. Conclusion

The practical use of Copan milk test, MRL β -lactam, and lactic coagulation test were investigated for the detection of β -lactams in milk. Both Copan and MRL β -lactam tests found ampicillin, amoxicillin and penicillin at or below the MRLs, but the lactic coagulation test could not detect above-mentioned β -lactams at or exceeding the safe level of MRL. Copan test and MRL β -lactam were easier to use than lactic coagulation test. MRL β -lactam test is more expensive and specific than two other tests, but less time-consuming; while Copan milk test have a broad spectrum of detection, so it could be applied for a

wide range of inhibitory substances in milk that is very important in the dairy industry. Lactic coagulation test was not sensitive to all three β -lactams at or exceeding the level of MRL. Therefore, it cannot be applied as a screening test in the dairy industry.

Conflict of Interests

Authors have no conflict of interest.

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