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Evaluation of the *Bacillus calidolactis* plate for post screening assay of β-lactam antimicrobial residues in Kenyan dairies

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Abstract

As part of efforts to establish a control program for antimicrobial residues in local Kenyan dairies, a low cost *Bacillus calidolactis* test plate was evaluated after laboratory standardization. Test parameters studied were: the practicability of the test handling, the test sensitivity in terms of limits of detection (LODs) compared to codex alimentarius maximum residue limits (MRLs) and repeatability within two different laboratories. The observed LODs were: penicillin G (2 μg/kg), ampicillin (2 μg/kg), amoxicillin (2 μg/kg), oxacillin (15 μg/kg), cefalexin (50 μg/kg), cephapirin (30 μg/kg) and ceftiofur (50 μg/kg). These levels were lower than established Codex Alimentarius MRLs. The agreement between the two laboratories was calculated to be 0.83 (83%) with a determined proportion of kappa (κ) ranging between 0.61 and 0.80κ which corresponds to a good agreement strength. The standard error se $(k) = 0.052$ and the confidence intervals at 95% for κ =0.51–0.71 for the two laboratories. The study suggests that raw milk exhibiting inhibition diameters of <19 mm ($p < 0.05$) in the *B.calidolactis* test plate fulfills the codex alimentarius MRL for the tested β-lactams at their various detection levels. It was concluded that the plate test could be a useful and affordable post screening assay for commonly used β-lactam antimicrobial in the dairy sector within low-income countries.

Keywords: Milk safety; *B.calidolactis* plate; β-lactam residues; Maximum residue limits; Screening assay

1. Introduction

In many countries a wide range of β-lactams has been approved for use in dairy cows. (Bruno, Curini, Di-Corcia, Nazzari, & Samperi, 2001; Verdon & Couedor, 1998; Zhi, Meyer, Van den Bedem, & Meusel, 2001). Six (6) beta lactams (penicillin, ceftiofur, cloxacillin, cephapirin, amoxicillin, and ampicillin) are widely used in treating disease in lactating dairy cattle and are the most likely to cause a residue in milk if misused.

In Kenya estimates indicate that approximately 14,600 kg of active antimicrobials are annually used in food animal production (Mitema, Kikuvi, Wegner, & Stohr, 2001) of which β-lactams are used extensively (Shitandi & Sternesjö, 2001). In the absence of control programs, as is the case in many low-income countries, usage of antimicrobials in lactating animals may lead to harmful residues in milk with subsequent problems as far as hygiene and the public health are concerned (Lin, Hong, & Kondo, 1995; NRC, 1999; Miller, 2002).

Maximum residue limits (MRLs) and withdrawal periods have thus been set in many countries worldwide. The limits are monitored in control programs, to minimise risks from the residues in human diet. One of the main obstacles to widespread application of a control program in the dairy industry in low-income countries is that it requires a considerable financial investment. The need for an inexpensive method of monitoring residues prompted this work and led to investigating a simple and reliable procedure that would be applicable in the local dairies. This study aimed at evaluating a developed *Bacillus calidolactis* plate (Rikilt-dlo, 1998.) as a post-screening test for β-lactam residues at or near established MRLs in Kenyan milk.

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2. Methods and materials

2.1. Samples

Antibiotic free milk from dairy animals with known history of no antibiotic treatment was collected from the Egerton University farm (Njoro, Kenya) for preparation of standards and spiked milk samples. As a further precaution the milk was screened with the Delvotest SP (DSM Food Specialties Dairy Ingredients, Delft, The Netherlands) as per manufacturer. All milk samples were defatted by centrifugation and stored frozen at −20 °C and used within one month.

2.2. Analytical procedure

2.2.1. Propagation of B. Stearothermophilus Var. Calidolactis C 953

A loop of the original *Bacillus stearothermophilus var. calidolactis C 953* spore suspension (supplied from Rikilt dlo laboratories, the Netherlands) was inoculated into 10 ml of nutrient broth (Difco, Detroit, MI, USA), and incubated at 55 °C for 12 h. The resulting bacterial suspension was spread on nutrient agar (Difco, Detroit, MI, USA) supplemented with 31.3 mg/l medium manganese sulphate ($MnSO_4H_2O$), analytical grade, (Merck, Darmstadt, Germany) for improvement of sporulation. The Petri dishes were incubated in a plastic bag (for prevention of drying) at 63 °C for 3 days. The spores were harvested using a scalpel, washed with physiological saline solution (0.85% NaCl) and centrifuged three times at 2700 rpm for 10 min. The suspension was then heat treated at 80 °C for 10 min to kill the bacterial cells and the final spore concentration was counted on plate agar (Difco, Detroit, MI, USA) incubated at 55 °C for 24 h.

2.2.2. Preparation of milk standards

Standard stock solutions of different β-lactams antimicrobial were used as: penicillin G, ampicillin, amoxicillin, oxacillin, cefalexin, cephapirin and ceftiofur. All were purchased from (Sigma Chemical Co. St. Louis, Missouri, USA).

From the stock solutions appropriate working solutions were prepared at the selected antimicrobials in ratios (0.25, 0.5. 1.0, 1.5 and 2.0) of the established MRL for each antimicrobial. Milk samples (10 ml of each sample) were heated at 80 °C for 10 min to inactivate natural inhibitory substances and kill contaminating bacteria. The samples were then allowed to cool to room temperature. Working solutions (0.1 ml) containing selected spiked antimicrobials were added to raw antimicrobial free milk (9.9 ml), and tested within 3 days. The analysis on each test plate included one blank sample and reference milk standards as described (Nouws et al., 1999; Rikilt-dlo, 1998). The spiked samples were randomized, coded in order to perform blind coded analysis and availed to the two laboratories.

2.2.3. Plate composition and incubation conditions

The concentration of microorganism, media composition and incubation conditions are shown in Table 1. For preparation of test plates the media was sterilized by autoclaving, cooled in a water bath to about 50 °C and inoculated with *Bacillus stearothermophilus var. calidolactis C 953* spore suspensions. The pH was adjusted to 8.0 by 1 M NaOH. The media was mixed and approximately 35 ml poured onto test plates of 24.5×24.5 cm (Gibco Europe Ltd., Breda, The Netherlands). The plates were covered, left at room temperature (22 °C) for about 15 min, and were then immediately used. After solidification of the agar, seven 14-mm diameter holes were punched out on each agar plate, the holes being distanced at least 30 mm from each other.

2.2.4. Plate assay procedure

To verify the detection limits the fortified milk samples with known concentrations of selected β-lactams antimicrobials were evaluated. Milk samples (250 μl) were pipetted into the holes and the plate was incubated at 55 °C/6 h. Each concentration was analyzed in 6 replicates and the trials repeated 30 times at each concentration over a five day period. The diameters of the inhibition zones (inclusive of the punch hole) were measured immediately after incubation with a vernier calliper.

3. Results

The results for the spiked milk samples are shown in the Table 2. The blank control milk samples gave no inhibition zones on any of the six plates. Table 2 shows that raw milk exhibiting inhibition diameters of <19 mm in the *B. calidolactis* test plate fulfills the codex alimentarius requirements for the selected β-lactams

Table 1
Composition of the β-lactam plate and incubation conditions

Test plate/Strain	Antimicrobial target	+ive control (μg/l)	Cfu/ml	Medium	pH	Buffer and supplement	Incubation temp/time ° C/h
B. stearothermophilus var. calidolactis C953	β-lactams	Penicillin G at 3 μg/l	10^7 spores	Plate count agar	8.0, adjusted with 1M NaoH	Nil	55 °C/6h