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Full Length Research Paper

Laboratory evaluation of the improved tube test detection limits for β-lactam residues in Kenyan milk

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In Kenya there is currently no screening of antimicrobial drug residues in milk. This study evaluated the improved tube test as a possible screening method using seven representatives of the β-lactam antibiotics. The group comprises antimicrobials most frequently used to treat bacterial infections in dairy cows. Each antimicrobial was tested at five concentrations based on established codex alimentarius maximum residue limits (MRLs). Test parameters studied were practicability and limits of detection (LODs) compared to MRLs and repeatability. The LODs established using a logistic regression model were: penicillin G (2 µg /kg), ampicillin (2 µg /kg), amoxicillin (2 µg /kg), oxacillin (30 µg /kg), cefalexin(100 µg /kg), cephapirin(60 µg /kg) and ceftiofur(100 µg /kg) all within codex alimentarius MRLs. The cost per ten samples using the improved tube test was less than 1 USD compared with 5 USD for the Delvo test. The improved tube test is concluded to be an affordable method, which could be used for qualitative identification of residues in low-income countries dairies.

Key words: Improved tube test, β-lactams, Kenyan milk, maximum residue limits.

INTRODUCTION

Antibiotics residues in bovine milk are a problem in Kenyan milk (Ombui et al., 1995; Shitandi and Sternesjö, 2001). The β-lactam group of antimicrobials are in particular commonly utilised in lactating animals (Mandell and Perti, 1996; Mitchell et al., 1998). They are consequently the most frequent contaminants in milk. It is important to avoid these residues because of toxicological and public health reasons (Honkanen and Reybroeck, 1997). In many regions of the world mandatory testing are carried out to determine drug violations (Suhren, 1995; Sternesjö and Johnsson, 1998). The tests used include microbiological, spectrophotometric, thin-layer chromatographic and bioautographic, liquid chromatographic, high-performance liquid chromatographic, gas chromatographic, mass spectrometric, and immunochemical methods (Nakazawa et al., 1992; Honkanen - Buzalski and Reybroeck, 1997; Elliott et al., 1998). In the East African region there are at present no such tests on supplied milk. The main reason for not incorporating such tests in a control program is because they are expensive and cannot be sustained by the local dairy industries.

The present study is part of on going efforts to evaluate possibilities of establishing a low cost microbiological test in a milk control system to prevent residues of β-lactam

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Table 1. Composition of the two test and incubation conditions.

Test plate/Strain	Antimicrobial target	+ive control (∝g /l)	Cfu/ml	Medium	pH	Supplement	Incubation Temp/Time ^{o}C/h
B.*stearothermophilus var. calidolactis C953*	Qualitative – Broad spectrum	Tube A Penicillin G at 1 *ug/l* Oxyteracycline 100 µg/Kg	10^{7} spores	Plate count agar	7.0	Chloramphenicol	63 ^{0}C/12h
		Tube B Sulphamethazine 100µg/Kg Penicillin G 1µg/Kg Dapsone 2µg/Kg			8.0	Trimethoprim	63 ^{0}C/12h

antibiotics. The improved tube test is a microbial inhibitor tests developed to detect a broad spectrum of antibiotics (Rikilt-dlo, 1998; Nouws et al., 1999). It involves incubating the test organism (*Bacillus stearothermophilus* var. *calidolactis*) in the presence of the milk sample. The test organism is cultured in two tubes in the presence of nutrients and bromocresol purple as the indicator dye. Under normal conditions as the culture grows the dye color is changed from purple to yellow. If an antibiotic is present the organism is inhibited and the dye remains purple.

This study is aimed at evaluating the laboratory performances of the improved tube test method as a screening assay for commonly used β-lactam antimicrobials based on established codex alimentarius (1993 and 1994) MRLs.

MATERIALS AND METHODS

Milk samples

Milk samples were collected from the Egerton University Njoro (Kenya) dairy farm and initially screened with the Beta STAR test (UCB Bioproducts, Belgium), which involves a specific β-lactam receptor linked to gold particles. From the beta star screening test, samples determined as negative were used in subsequent analysis. The milk representative samples with a chemical composition and pH values considered normal for Kenyan local dairies as routinely tested at the Guildford dairy plant (Egerton University Njoro, Kenya).

Screening tests

The composition of the improved tube test and incubation conditions are provided briefly in Table 1. A comprehensive description of the test conditions, culturing of the *Bacillus stearothermophilus var. calidolactis C 953* spores, reagent preparations and methodology used is described in the protocol of the method by Rikilt-dlo (1998) and Nouws et al., (1999). The requirements for sporulation of *B. stearothermophilus var. calidolactis* were optimized in this study by supplementing the media with 50 ppm $MnC_{l2}{\cdot}4H_{2}O$ (incubation 24 ± 2 h/ 55 °C). The spore cultures, washed off the media with $MnCl_{2}$-solution, were boiled in a waterbath for 10 min before being used in Nutrient agar (heat activation). Improved sensitivity of the *B. stearothermophilus var. calidolactis* was further achieved by the incorporation of trimethoprim, chloramphenicol in the test medium at pH = 7.0 for one tube and trimethoprim with phenylbutazone at the pH 8.0 in the second tube.

The commercial Delvo test (Gist-brocades BV, The Netherlands) was used as the reference test and performed as described by the manufacturer. All the antimicrobials (analytical grade) were purchased from Sigma Chemical Co., St. Louis, MO (USA).

Assay procedure

The International Diary Federation (IDF) guidance for the standardized description of microbial inhibitor tests (IDF - Group E 503, 1997) principle of verification was followed. Each antimicrobial was tested at five different concentrations as: 0.25 x MRL, 0.5 x MRL, 1.0 x MRL, 1.5 x MRL and 2.0 x MRL with thirty replicates at each concentration. In every case a negative control and positive controls (according to claimed detection limits) is included. The β-lactams antimicrobials and concentrations used were: pencillin G (1.0, 2.0, 4.0, 6.0, 8.0 µg /kg); ampicillin (1.0, 2.0, 4.0, 6.0, 8.0 µg /kg), amoxicillin (1.0, 2.0, 4.0, 6.0, 8.0 ug /kg), oxacillin (7.5, 15, 30, 45, 60 µg /kg), cefalexin (25, 50, 100, 150, 300 µg /kg), cephapirin (15, 30, 60, 90, 120 µg /kg) and ceftiofur (25, 50, 100, 150, 300 µg /kg).

Adulterated samples as well as normal control samples were then randomized and numbered (blinded), such that the contents of the test samples were unknown to analyzers performing the assays. Each concentration was replicated 30 times by the two analysts, with analysis spread over 2 weeks. A total of 300 test samples were analysed for each antimicrobial. Two analysts in the two laboratories performed all the experiments to minimize inter assay variation. The code for the samples was broken and the performance data compiled for each of the two methods. In the method, scoring of a positive or negative result was based on a color chart, on a subjective scale ranging from one to five (one is negative and five is very positive). From the chart, 1 and 2 were thus negative, 3 was doubtful while 4 and 5 positive. In subsequent analysis doubtful scores were considered positive.

Table 2. Improved two tube test test (n = 30) ability to detect different β-lactams drug concentration at 0.5x MRL, 1.0xMRL, 1.5xMRL and 2.0xMRL.

B-lactams Antimicrobial	Codex MRL	Drug Concentration (∝g/kg)tested				
	∝g/ kg	0.25xMRL	0.5xMRL	1.0xMRL	1.5xMRL	2.0xMRL
Penicillin G	4	+/-	+	+	+	+
Ampicillin	4	+/-	+	+	+	+
Amoxicillin	4	+/-	+	+	+	+
Oxacillin	30	+/-	+/-	+	+	+
Cefalexin	100	ND	ND	+	+	+
Cephapirin	60	+/-	+/-	+	+	+
Ceftiofur	100	ND	+/-	+	+	+

+ means that the β -lactam antimicrobial was detected in 90% of the time with 95 % confidence. +/- means that the β -lactam antimicrobial was detected (Based on 30 samples at each concentration) in less than 90% of the time with 95 % confidence.
ND means that the β-lactam antimicrobial was not detected in any of the analysis

Table 3. β-Lactam detection levels of the improved two tube test after laboratory standardization as related to fulfilment of the maximum residue level (MRLs) allowed by codex alimentarius.

β-lactams Antimicrobial	Present study detection limits (LOD) ug/ kg	Codex MRLug/ kg	Commercial Delvo test SP	Fulfill (Yes/No)
Penicillin G	2	4	2	Yes
Ampicillin	2	4	2	Yes
Amoxicillin	2	4	2	Yes
Oxacillin	30	30	15	Yes
Cefalexin	100	100	50	Yes
Cephapirin	60	60	30	Yes
Ceftiofur	100	100	50	Yes

Statistical analysis

The obtained data being categorical, was tested using a logistic model (Agresti, 2002; Charles et al., 2001). In brief, the model was:

$$L_{ij} = \beta_0 + \beta_1 A_i + \sum ij$$

where L_{ij} is the variable logit, i.e. in $P_{ij}/1- P_{ij}$; P_{ij} is the probability of "positive response"; 1- P_{ij} is the probability of "negative response; β_0 , β_1 are the coefficients estimated for logistic regression models; A_i is the antimicrobial agents concentration and is the $\sum ij$ residual error. The results were determined using SAS (SAS Institute, 2001), logistic procedure. The concordance coefficient was used as a rank correlation considering the observed responses against the predicted probabilities. The detection limit of the improved tube test was estimated as the concentration in which 95% of the results obtained were scored as positive.

Agreement between the two analysts was considered to determine their ability to classify the samples into one of the several groups. The ability of the two analysts to reproduce results using the improved tube was investigated using the **C**ohen's kappa coefficient (k) as described by Aviva and Watson (1999). The k, measures agreement based on observed categorical data. In the method, scoring of a positive or negative result was based on a color chart, on a subjective scale ranging from one to five (one is negative and five is very positive).

RESULTS

Table 1, gives a composition of the improved tube test and incubation conditions. Among the heat activated spores, cultures showed the best results (microscopical: spore content about 50 %, highest plate counts/ml and shortest incubation period in the PCA) for the production of spore cultures with those whose media had been supplemented with 50 ppm $MnC_{l2}·4H_2O$ agar (Oxoid), a pH-value of 7.5 and an incubation temperature of 60 °C proved to be most effective. The results were observed to be stable for 8 hours after assay completion and could be read by visual color comparison or a color chart. The test was completed in approximately 5 hours and upto 100 samples could be be run simultaneously.

From Table 2, the ability of the improved tube test to detect different β-lactams was observed to be good as all the tested antmicrobials could be detected at the 1.0 x MRL in 90 % of the time based on 30 replicates at each concentration. At a lower limit of 0.5 x MRL only cefalexin was not detected in any of the analysis.

Table 3, gives results of the detection limits as relates to the fulfilment of the MRLs allowed by codex

alimentarius. The improved tube test was able to detect all the tested seven antimicrobials at the established MRLs. The exact agreement of the two analysts using the improved two tube test on a score of 1-5 for penicillin G results agreement based on a colour chart was calculated from Table 4, to be 0.63 (63%). Under the null hypothesis of no association the number of agreements expected by chance was 38.22 that gives a proportion of the total as 0.21 (38.2/180). The analysts' agreement as a proportion of kappa (k) is calculated to be 0.61. The results were interpreted following the guidelines from Aviva and Watson (1999), where a 0.61 - 0.80 k value corresponds to good strength of agreement. The results suggest that the strength of agreement between the analysts was good. The standard error (se (k)) and confidence intervals at 95% were calculated based on the obtained k as; se (k) = 0.052 and the 95% confidence interval for k = 0.51 to 0.71.

Table 4. A comparison of two laboratories penicillin G results agreement using the improved two tube test on a score of 1-5 based on a colour chart.

Score (total)	1	2	3	4	5
1(32)	**25**	6	1	0	0
2(20)	4	**10**	4	2	0
3(36)	0	2	**26**	7	1
4(38)	0	1	5	**23**	9
5(54)	0	0	3	21	**30**
Total (180)	29	19	39	53	40

Table 5, gives a summary of the results observed with both the tube test and the reference Delvo test used in the study. The sample throughput was good as over 70 samples could be analysed on a 12 hour basis by each of the analysts who were semi skilled. From the Table 4, the cost per sample of the tube test was observed to be 20 % cheaper than the commercial Delvo test and also the other indices compared (sensitivity, specificity and predictive value) at the cut off pencillin G limit of 4 ∝g/Kg, compared favourably with the commercial Delvo test.

DISCUSSION

The *Bacillus stearothermophilus* used as the test microorganism in this study is routinely used in commercial tests in many regions and the organism is reported to have good sensitivity to the group of β lactam antibiotics (Messer et al., 1982; Reybroeck, 1995; Suhren and Beukers, 1998; Jevinova et al., 2003). The validation of LODs for any developed method is however necessary even if the test organism is similar as other factors such as the test reagents, composition of milk and laboratory conditions tend to differ (IDF, 1997). The improved test was observed to be reliable and reproducible in its ability to detect the tested seven antimicrobials and also a cost effective qualitative procedure. A positive result from a screening test is however only a presumptive indication that a residue is present in the milk sample. The screening test does not necessarily identify the specific residue causing the test to be positive nor does it measure the quantity. Other methods would be required to determine whether or not a given milk sample contains antimicrobial drug residues above the tolerance/safe level.

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The observed LODs (Table 3) exhibited good agreement with the LODs in the methods description (Rikilt dlo. 1998; Nouws et. al., 1999). In a previous study (Shitandi & Sternesjö, 2001) the tube test had been observed to have a good sensitivity with the method having an LOD of 1.5∝g/Kg compared to the 2 ∝g/Kg

Table 5. Summary of the results observed with the improved two tube test and Delvo test.

Parameters	Improved two tube	Delvo Test SP
Quantitative/Qualitative	Semi-qualitative	Semi-qualitative
Sample throughput	>70/day	>70/day
Analyst	Semi-skilled	Semi-skilled
Sensitivity at 4µg/kg	96%	92%
Specificity at 4 µg/kg	92%	96%
Positive predictive value	94%	96%
Negative predictive value	95%	91%
Cost per sample	<1USD	<5USD

observed in this study, of which both results were within the established MRLs. The initial study however only investigated penicillin G and not other β-lactam family members and it also did not study inter analyst variation which is necessary in the validation of an assay. The present findings (Table 3) suggest that the two tube method can detect a wider range of residues of commonly utilised β-lactam family in Kenyan milk which compare favourably with the claimed LODs of the reference commercial Delvo test at 2.5 ∝g/Kg for penicillin G; 3- 4 ∝g/Kg for amoxicillin and ampicillin; and 5- 8 for cephapirin (Suhren and Beukers, 1998).

The analysts' agreement as a proportion of kappa (k) determined in this study showed a good strength of agreement. This was useful in determining reproducibility and interlaboratory comparisons for practicability of the test handling. The ability to grow the *B. stearothermophilus* spores in local conditions using semi skilled labour as undertaken in this study offers in particular, a low cost solution for detecting β-lactams. Further interlaboratory standardization would however be recommended before a standardized protocol can be distributed within a region. It would also be of future use to investigate the span of the two tube screening assay for other families of drugs such as sulfa drugs, tetracyclines, aminoglycosides and macrolides in raw, commingled, bovine milk.

The present Kenyan specifications for milk residues stipulates zero tolerance in raw milk, heat treated milk and products. This decision needs to be updated, as it does not work especially in the context of international guidelines referring to the concept of MRLs (Codex Alimentarius, 1993). A three-step strategy is required for residue control in food to be in conformity with established MRLs (Codex Alimentarius, 1993). This strategy allows a first qualitative assay (screening) to point out the samples postive of a generic inhibitory substance. An assay used for milk screening, especially in low income countries where the retail costs of milk are low, needs to be inexpensive, easy to perform and should be able to detect a broad spectrum of antimicrobials.

Factors normally considered in sele ction of a suitable method of residue detection are the type of antibiotic used, expected time limitations, sensitivity and costs (Senyk et al., 1990). The two tube method was observed to be sufficiently sensitive and specific to fulfil the requirements of codex alimentarius. Minimal use of capital equipment is needed for the test, as the main requirements are an incubator, refrigerator and a water bath. Although exact quantification of antibiotic concentrations cannot be assessed with the tube test method, a semiskilled analyst can reliably classify milk samples as positive or negative for common members of the β-lactams that exceeds established MRL. The improved tube test could thus be a useful method for analysis of raw milk in dairies in low-income countries. In such a program a farmer quality payment scheme would be implemented where bulked samples will be tested weekly in retrospective.

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