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Assessment of the California mastitis test usage in smallholder dairy herds and risk of violative antimicrobial residues

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This study evaluated how predictive the California Mastitis Test (CMT) is for sub-clinical mastitis under tropical smallholder dairy production conditions in Kenya. It intended to establish whether the CMT usage could be contributing to misdiagnosis and consequent mistreatment with animal drugs resulting in residue problems. Milk samples (n = 239) were aseptically collected from lactating cows in the Rift Valley of Kenya and tested using the CMT, somatic cell counts (SCC) and bacterial culture. The samples were also screened for violative drug residues using the commercial delvo test and compared to the milks mastitic status for possible association. There was a numerical but non-significant ($p > 0.05$) difference evident in the frequencies observed using the three different mastitis indicators. The prevalent bacterial species isolated from mammary glands with subclinical mastitis were *Staphylococcus aureus* (45.6%), coagulase-negative Staphylococci (13.0%), Streptococci (11.7%) and *Escherichia coli* 5.9%. There was an overall poor but significant ($p < 0.05$) correlation between the CMT and the violative antimicrobial residues in samples from all quarters, infected and non-infected respectively. The results suggest that the CMT use amongst the smallholder dairy sector as a mastitic indicator may not be a risk factor in violative antimicrobial residues problems in milk.

Key words: California mastitis test, mastitic indicator, antimicrobial residues, milk

Introduction

In lactating dairy animals the usage of antimicrobials is often aimed at treatment of mastisis an important diseases in dairy herds [14]. The disease is usually classified into two forms, clinical in which the disease is diagnosed visually or by palpation, and subclinical which is mainly diagnosed via assessment of somatic cell and/or bacterial culture [1,2].

Veterinarians and milk producers use various mastitis indicators to help guide treatment decisions [5,4] although information on mastitis screening tests and their relevance for application in tropical conditions are scanty. The California Mastitis test, (CMT) is one such test utilized widely to determine the disease status of lactating animals, as it is simple, inexpensive and rapid screening test. The CMT reagent reacts with material from the nuclei of the somatic cells in the milk to form a gel and it estimates the number of somatic cells present in milk. The reaction is then visually scored, with interpretation dependant upon the amount of gel that forms [8].

In Kenya, many smallholder farmers being resource poor opt to use the California Mastitis Test (CMT) to detect subclinical mastitis infection. They do not take milk cultures to diagnostic laboratories, as there is widespread lack of diagnostic services due to the costs involved and limited laboratories. On the basis of a positive CMT score farmers then use antimicrobials for therapeutic treatment of the disease. The use of CMT is based on the increased number of somatic cells in milk and udder. There is however some evidence that the number of somatic cells in milk is relatively higher under tropical dairy production conditions, especially smallholder dairying [12]. This is attributable to poor hygienic conditions and high incidences of infections common in tropical dairy production systems. It was thus hypothesized that under smallholder dairy production conditions, positive sub-clinical mastitis based on CMT results may not be good indication of the presence of bacteria that cause udder infections. The study thus intended to establish whether the CMT usage could be contributing to misdiagnosis and consequent mistreatment with animal drugs resulting in the residue problems as evident from a previous study [19]. The research question of interest was how predictive the California mastitis test (CMT) is under smallholder dairy production conditions, for the presence of

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pathogenic bacteria that cause sub-clinical mastitis.

Materials and Methods

Milk samples

A cross sectional study was done in the Rift Valley, a major milk production area of Kenya. Milk samples from 239 quarters (from 45 herds, which were crosses of Holstein Fresian and local breeds), were obtained by stratified random sampling. Milk samples were collected from the same herds at 1-month intervals until the calves were weaned. The samples were collected while the cows were restrained in a standing position. Udder and teats were cleaned with water and left to dry and then were swabbed with cotton soaked in 70% ethyl alcohol. The first three streams were discarded before 5 ml of milk were collected in sterile containers. Milk samples were initially screened with the CMT test reagent and then transported to the microbiological laboratory, Egerton University on ice and tested within 6 h of collection.

CMT

The CMT reagent (DeLaval, Wroclaw, Poland) was used and the test was carried out using the method described by [17]. The reaction was then visually scored as 1, 2, 3, 4 or 5 according to the Scandinavian recommendations [7] depending upon the amount of gel that forms. The reaction was interpreted as follows: score 1 = no reaction; score 2 = slight slime which disappears with continued swirling; score 3 = distinct slime but without gel formation; score 4 = immediate formation of gel which moves as a mass during swirling; score 5 = gel develops a convex surface and ad hears to the bottom of the paddle.

Somatic cell count

Somatic cell counts (SCC) were determined by spreading 10 µl of thoroughly mixed milk from each sample over 1 cm^2 area on a glass slide. The slides were left to air-dry and were stained by Newman-Lampert stain as described by [6]. SCCs of = 10^5 cell/ml milk were considered to be positive.

Bacteriology

Isolation and identification were carried out according to the Scandinavian recommendations on examination of quarter milk samples [9]. In brief, immediately after delivery, the milk samples were inoculated on blood agar plates (Becton Dickinson and Company, USA), which were divided into four sections. A disposable culturing loop (10 µl) was dipped into the milk sample and a total of 6-8 lines made in one agar section by turning the loop once between the streaking lines. Each quarter milk sample (10 µl) was thus streaked in one section of the plate as a line culture. Samples were cultivated under aerobic conditions for 24-72 h at 37°C and examined for bacterial growth. Pure cultures were further examined for morphological, staining and cultural characteristics, and for biochemical reactions. In cases of mixed growth, a new quarter sample was taken and reexamined. Where no growth was detected, plates were reincubated at 37°C for an additional 24 h. Bacteria were identified using a standardized procedure [10]. API 20 Strep and API Staph (BioMérieux, Basingstoke, Hants) were used for speciation of Streptococci and coagulase-negative *Staphylococcus*. When fungal infection was suspected, portions of the milk were cultured on Sabouraud dextrose agar (Difco, Detroit, MI, USA).

Quarters were classified as not infected if no organisms were isolated and infected with major pathogens if *Streptococcus agalactiae*, other *Streptococcus* species, *Staphylococcus aureus*, and *Escherichia coli*, coagulase-negative *staphylococci* were isolated. Quarters were also classified as "normal" if no organisms was isolated, the udder had no injuries or indurations, the appearance of the milk was normal, and no previous history of mastitis was recorded and "abnormal" otherwise. Samples with unspecified mixed cultures were considered contaminated and thus excluded from subsequent analysis.

Violative antimicrobial residues

All samples were examined the same day for the presence of antimicrobial residues. The milk samples were all preheated at were pre-heated at 80°C for 10 min to inactivate natural inhibitory substances and kill contaminating bacteria. The commercial Delvotest SP (GistBrocades, Delft, the Netherlands) test was then used to screen the samples which was performed as per the manufacturer and included appropriate positive and negative control samples in all assays. The test is a microbial inhibitor test, which utilises *Bacillus stearothermophilus* as the test organism with a dye in the media and detects antimicrobial substances non-specifically. 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.

Statistical analysis

For comparison of the methods (SCC, CMT and bacteriological), statistically significant association was determined by the Chi-squared test, based on cut off for results as positive or negative. All statistical analysis was performed using the minitab, 2000 program [9]. Statistical significance was set at the 5% level. Data was assessed by least square analysis of variance, using the general linear model procedure. The model included the effects of the animal, the quarter, the stage of lactation (1-12 months), the bacteriological findings and the antimicrobial status. The results are presented as means ± SEM. The correlation coefficient between the mastitic indicator-CMT and

Table 1. Observed frequencies (%) of milk samples (n = 239) positive and negative for mastitis using the CMT, SCC, and bacteriological mastitic indicators from apparently unaffected cows

	CMT	SCC[a]	Bacteriological
Negative	39 (16.3%)	48 (20.1%)	57 (23.8%)
Positive	200 (83.7%)	191 (79.9%)	182 (76.2%)

[a]SCCs of $>10^5$ cell/ml milk was considered positive
P value > 0.05

Table 2. Bacteriological findings (%) in quarter samples (n = 239) from smallholder cows

Infection Status / Proportion N (%)	Non infected	*Staphylococcus aureus*	Coagulase negative staphylococci	*Streptococci spp*	*Escherichia coli*
Proportion (%)	57 (23.8)	109 (45.6)	31 (13.0)	28 (11.7)	14 (5.9)

Table 3. †Mean ± SEM of SCC and CMT for infected and non infected quarters of cows

Component	n	Infected ($\times10^5$)	Non infected ($\times10^3$)	P-value
SCC	186	18.24 ± 0.24 (n = 148)	20.05 ± 0.39 (n = 38)	$p < 0.01$
CMT score	239	1.98 ± 0.14 (n = 18)	23.49 ± 0.29 (n = 57)	$p < 0.001$

†The data are presented as mean cells per milliliter ± standard error of the mean for cows in each group.
SCC = somatic cell count; CMT = California mastitis test; n = number of observations.

Table 4. †Mean ± SEM of SCC and CMT in non -infected and in S. aureus and CNS -infected quarters of cows

Component	N	*S. aurues* ($\times10^5$)	Non infected ($\times10^3$)	CNS
SCC	186	18.24 ± 0.24 (n = 109)	19.53 ± 0.59 (n = 46)	19.47 ± 0.89 (n = 31)
CMT score	209	1.98 ± 0.14 (n = 105)	3.11 ± 0.36 (n = 81)	3.38 ± 0.30 (n = 13)

†The data are presented as mean cells per milliliter ± standard error of the mean for cows in each group.
SCC = somatic cell count; CMT = California mastitis test; CNS = coagulase negative staphylococci
n = number of observations.

violative antimicrobial residues (as measured by the Delvo test SP) was calculated using Spearman rank correlation of the residual obtained after correcting for the effects in the statistical model.

Results

Observed frequencies of milk samples from apparently unaffected cows, which were positive and negative for mastitis using the three mastitic indicators (CMT, SCC and bacteriological), are displayed in Table 1. While there was a numerical difference evident in the frequencies observed using the mastitis indicators the difference was not significant (p value = 0.121 > 0.05).

From Table 2, intramammary infections were present in 76.2% of the 239-quarter milk samples examined. *S. aureus* and Coagulase negative staphylococci (CNS) represented 59.9% and 17.0% of the isolates respectively with *Streptococci* representing 15.4%.

The mean values for the SCC and CMT from the infected and non-infected quarters are shown in Table 3. Infected udder quarters had significantly ($p < 0.01$) higher mean values for both SCC and CMT. Eleven of the 239 individual quarter samples had somatic counts >5,000,000/ml which was considered very elevated for apparently normal milk. There were also eight samples of the 239 individual quarter samples that showed high values for CMT and SCC from which bacteria were not isolated.

Table 4, shows separately the mean values for non-infected quarters and quarters infected by CNS and *S. aureus.* From the statistical model, the correlation coefficient between the mastitic indicator-CMT and violative antimicrobial residues (as measured by the Delvo test SP) was calculated using Spearman rank correlation. The correlations from all quarters was 0.59 (n = 186); infected quarters had a correlation of 0.38 (n = 148) and non-infected was 0.48 (n = 38). All positive and negative control samples yielded the expected reactions and >12% of the milk samples collected from the herds contained inhibitory substances which were heat stable. There was an overall poor but significant ($p < 0.001$) correlation between the CMT and the violative antimicrobial residues.

Discussion

The relationship between the mastitis indicators, CMT, SCC and bacterial culture was investigated based on the proportions of positives at set cut off limits. The null hypothesis was that the true mastitis rates are the same for the three indicators with the alternative hypothesis being that they are not equal. The results suggest that under smallholder dairy production conditions, positive sub-clinical mastitis based on CMT results may be good indication of the presence of bacteria that cause udder infections. The CMT may thus not be contributing to misdiagnosis and consequent mistreatment with animal drugs resulting in the residue problems.

While the spearman's rank correlation coefficient provided a measure of association between the two variables it does however not imply causality, although in some studies [15,16] an association has been observed between measures of milk quality and risk of violative antimicrobial residues.

There are however many factors that contribute to the overuse of antimicrobials in farming which arises from a complex interaction between the cow, microbial agents, environmental influences and management factors [18]. The Delvotest microbial inhibitor test can be inhibited by lysozyme or lactoferrin separately and synergistically [3]. These inhibitors and other non-protein inhibitors occur frequently in high SCC (>4,000,000/ml) milk and could be a problem in tropical conditions where SCC has been observed to be abnormally high [12], a finding also observed in this study. The inhibitors are however heat labile and hence the need to heat-treat the samples at >80°C as was done in this study.

A critical aspect in the evaluation of screening tests is the criteria of deciding upon the true status of the udder. In previous studies, bacteriological findings, SCC, or a combination has been used [5,7]. *S. aureus* is regarded in many regions as a major causative organism for mastitis [11,13] and this was found to be the case in the study site justifying its possible use as a true status of udder infection within the region.

The field study provided no clear evidence that CMT could be less predictive compared to the other two mastitis indicators in the small herd sector. The test is thus unlikely to be contributing to misdiagnosis and consequent mistreatment with animal drugs resulting in the violative residue problems inherent in milk. There were however samples that showed high values for CMT and SCC from which bacteria were not isolated and this together with the apparently high rate of intramammary infection in the smallholder dairy sector warrants further investigation. In the model that was used to determine possible association between CMT usage violative residue problems although the observed correlation was weak, the observed frequencies of antimicrobial residues emphasize the importance of screening farm milk for antibiotic residues.

In conclusion the results suggest that the CMT may be a useful indicator of udder infection on farm that should however be used alongside bacteriological culture. A program is also needed in the region, designed to improve milk quality and udder health so as to prevent new intramammary infections, eliminate existing infections and monitor udder health status.

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