International Scholars Journals

African Journal of Dairy Farming and Milk Production ISSN 2375-1258 Vol. 3 (4), pp. 152-158, April, 2016. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full length Research Paper

Analysis of antibiotic residues in milk from smallholder farms in Kenya

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Received 18 December, 2015; Accepted 18 February, 2016

The aim of this study was to determine the occurrence of antibiotic residues in Kenyan smallholder farm milk using screening tests, and to identify the antibiotic residue group. A total of 480 milk samples were analyzed. All samples were analyzed with the Delvotest® screening test. A Hundred and fourteen (24%) samples were positive, 71 (15%) unclear and 295 (61%) negative. Sixty-two samples were further tested with the group specific Trisensor test. Twenty four percent (15/62) were positive. This indicated that by estimation, 9% of all the 480 samples have been positive with the Trisensor test and 5% would have contained beta-lactams, 2.5 % sulfonamides and 0.6 % tetracyclines. Samples with a positive Trisensor test results were further analyzed with HPLC but no antibiotics could be identified. Seventy six percent of the Delvotest® positive samples were negative in Trisensor test. Microbiological inhibitor methods are demanding for the sample conditions and were found not to be best suited to the conditions encountered in smallholder farms in Kenya. The results indicate that antibiotic residues are found in milk produced on small scale farms in Kenya and suggest that training is needed on the use of veterinary drugs.

Keywords: Antibiotic residues, milk, Kenya, Delvotest®, Trisensor, screening tests, sulfonamides, tetracyclines, beta-lactams, HPLC.

INTRODUCTION

Antibiotic residues in cow milk are a great concern, not only in developed countries with systematic residues detection programs, but also in developing countries where most of the milk bypasses official quality assurance channels creating a potential public health risk (Aboge et al., 2000). The antimicrobial usage in food production animals worldwide is estimated to increase even by 67% between 2010 and 2030 and this increase will be even higher where production is shifting towards large scale farming (Van Boeckel et al., 2015).

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Antibiotic residues in Kenyan milk have been analyzed along the market chain (Aboge et al., 2000). Besides lack of adherence to withdrawal times after antibiotic treatment, residues can be found if animals ingest feed contaminated with antibiotics (Aboge et al., 2000, Kang'ethe et al., 2005). Kang'ethe et al. (2005) found that consumers of milk and dairy products are five times exposed to products with antibiotic residues compared to 11 times per month for consumption of the same in Tanzania (Kurwijila et al., 2006).

Aboge et al. (2000) found that antibiotic residues in milk samples were three times higher in rural areas compared with urban areas. Other studies have indi-

cated similar results (Kang'ethe et al., 2005). They also found that the level of antibiotic residues at consumer level was higher than on market level, being 9.4% and 5.7%, respectively. The increase in the number of antibiotic residue positive milk samples along market chain implies that antibiotics could be added to milk on purpose (Aboge et al., 2000); alternatively this might reflect bulking processes along the chain. In the study of Ekuttan et al. (2007) the antibiotic residue prevalence in Kenyan urban farm milk samples was 4%.

Data from veterinary antibiotics used for mastitis treatment or dry cow therapy are not available in most developing countries (IDF, 2010). In Kenya, aminoglycosides, beta-lactams, sulfonamides and tetracyclines are most commonly used in the treatment of livestock (Abogeet al., 2000). Drug residue findings above the residue limits have increased in Kenya since the market liberalization. In 1978 penicillin was found only in 1% of the milk samples (Kang'ethe et al., 2005) whereas in 2000 the drug residue was found in 16% (Omore et al., 2004, Kang'ethe et al., 2005). Results from such a long time period should be taken with reservation; the increase in findings can be a result of improvement in sensitivity of analytical methods than increase in drug misuse resulting in more positive samples.

Shitandi and Sternesjö (2004) studied farm practices related to veterinary drug usage. Only 22% of the small-scale farmers documented drug usage, whereas on large-scale farms documentation was available in 74% of the farms. According to the study of Mitema et al. (2001) tetracyclines were the most used antibiotic group (55% of farmers), sulfonamides second by 21% and beta-lactams third by 6% share.According to the risk assessment tool done by Shitandi and Sternesjö (2004) lack of educational training in antibiotics use and their ill effects among farmers has been considered as one of the main reasons for antibiotic residues occurrence in Kenyan smallholder farm milk.

In antibiotic residue screening studies EU,(European Union) and Codex regulations for MRLs (Maximum residue levels) are mainly followed. The sum of sulfonamides should not exceed 100 µg/kg (EUR-lex, 2010). The MRLs for tetracyclines are 100 µg/kg (EUR-lex, 2010 and Codex, 2012). The MRLs for beta-lactams vary by compound, but mainly are below sulfonamide and tetracycline limits.

The data of antimicrobial usage on livestock and sales data is still non-existing in middle- and low-income countries (Van Boeck et al., 2015) and screening of the residues in milk is needed. The aim of this study was to determine the occurrence of antibiotic residues in farm milk sampled from Kenyan smallholder farmers in two selected areas using rapid tests for screening and HPLC for confirmation.

MATERIALS AND METHODS

Milk Sample Collection

The survey areas (Nandi and Makueni) were identified in 2009 during the preparatory phase of the project. The study area selection was based on evidence of high rates of cancer associated with consumption of mycotoxin contaminated maize and history of acute aflatoxicosis. Household selection was based on the following criteria: a household had to have at least one milking dairy animal, cultivate maize and have at least one child under five years old. Sample size determination in both sites was based on the number of qualifying households and population of the area. Results for the mycotoxin levels from the study households will be presented in separate publications.

A total of 480 milk samples were collected from individual small-scale farms in two counties of Kenya, Nandi and Makueni, during the autumn of 2010. Milk samples were collected directly from the household respondent's container and transferred to 500 mL plastic bottles. Collection continued throughout the day in conjunction with a household interview. Milk samples were placed with ice packs in a cool box and later frozen before transportation to the University of Nairobi laboratory once a week in cooled boxes. Aliquoted milk samples were kept frozen until analyzed.

Screening Tests

Delvotest® screening

Delvotest® SP-NT (DSM Food Specialties, Delft Netherlands) is a standard diffusion test for detecting antimicrobial substances, such as antibiotic residues, from milk. Positive sample would be indicated by inhibition of growth of *Bacillus stearothermophilus* while a negative sample would be indicated by growth of *B. stearothermophilus* and change of the medium test the color from violet to yellow. Activation of the test needs incubation for three hours at 64 °C degrees in a water bath or in an oven (Delvotest® instructions).

The Delvotest® screening was carried out as per the instructions of the manufacturer (Delvotest® instructions). Milk samples were thawed at room temperature. A total of 480 samples were analyzed, one sample set included 10 – 50 samples. Analysis was performed by adding 100 µl of milk sample to the test ampoule and incubating for 3 hours at 64 °C with a negative and a positive control in an incubation oven without water bath. The results were read immediately after 3 hours incubation.

Trisensor screening Test

The Trisensor (Unisensor, Liège, Belgium) test is a competitive assay in dipstick format based on two specific receptors and one antibody, which are able to detect residues from three antimicrobial groups; sulfonamides, tetracyclines and beta-lactams. The Trisensor test was used to identify the cause of the positive and unclear results of Delvotest® screening. Altogether 62 samples were analyzed due to the

limited number of test kits. The milk samples were thawed at room temperature. The positive and negative controls were included. The test was carried out following the manufacturer's instructions strictly.

High-Performance Liquid Chromatography (HPLC) analysis

The multi-residue HPLC analysis for milk was based on the method described by Mamani et al. (2009). In sample preparation, some modifications were applied from the study of Koesukwiwatet al. (2007).

The multi-residue HPLC method was developed to detect 4 tetracyclines; tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) and 7 sulfonamides; sulfamethoxazole (SMX), sulfadimidine (sulfamethazine) (SMTZ), sulfachloropyradizine (SCL), sulfamerazin (SMR), sulfathiazole (STZ), sulfadoxine (SDOX) and sulfadimethoxine (SDM) and chloramphenicol (CLF).

Equipment

The chromatographic column was XTerra MS C_{18} (2.1 x 150 mm, 3.5 µm, Waters) with an XTerra guard column MS C_{18} (2.1 x 10 mm, 3.5 µm, Waters). The method was validated with an equipment consisting of HPLC model Alliance 2690 Separation Module by Waters and Photodiode array detector, 2996(Waters Corporation, USA). In analyses of samples, the equipment comprised of a ShimadzuUV –detector SPD-20A, pump module LC-20 AT and an auto-sampler SIL-20A (Shimadzu Corporation, Japan). Software for the equipment was Shimadzu LC solutions.

Standards and reagents

All antibiotics were purchased from Sigma-Aldrich (Sigma-Aldrich Corporation, USA). Other reagents were from J.T. Baker (USA), Merck (Merck & Co., Inc., USA) and Panreac (Spain). The mobile phase gradient was composed of component A (0,075 M sodium acetate, 0,035 M $CaCl_2$, 0,025 M sodium EDTA (Ethylenediaminetetra-acetic acid), pH 7) and component B (methanol: acetonitrile, 75:25). Standard stock solution for the antibiotics was prepared in methanol (1 mg/mL) and was further diluted in distilled water for a concentration of 10 µg/ml (working solution).

Sample preparation

Milk sample preparation included extraction, purification and evaporation. Milk sample (5 ml) was mixed with TCA (Trichloroacetatic acid) (25%, 2.5 ml) in a centrifuge tube (25 ml) for 10 s with vortex. McIlvaine-EDTA buffer (10 mL, 0.1 M Na-EDTA, 0.1 M citric acid, 0.2 M Na2HPO4, pH 4) was added and the sample was vortexed for 10 s, ultra-sonicated for 10 minutes and centrifuged at 4000 rpm at 10 °C. Samples were cooled before centrifugation in a freezer -18 °C for 30 minutes. The clear supernatant was poured to anothercentrifuge tube (25 ml) leaving the fat layer in the first tube. The extraction with McIlvaine-EDTA buffer was repeated and the clear supernatants were combined.

Milk sample extracts were purified with Oasis HLB solid-phase extraction (SPE) cartridges, (C_{18}, 6cc, 200mg, Waters Corporation, USA). The cartridges were activated with 5 ml methanol, 10 ml of acetonitrile and 5 mL of McIlvaine-EDTA. 20 minutes was used to load the sample extracts through the cartridges. The cartridges were washed with 10 mL of 5% methanol in McIlvaine-EDTA and then dried with vacuum for 5 minutes. The antibiotics were eluted with 5 ml of methanol. The eluent was dried in a nitrogen flow in a warm water bath (40-50 °C) and the residue was dissolved in component B of the mobile phase (200 µl), vortexed and then mobile phase component A (300 µl) was added.

HPLC Analysis

Sulfonamides were detected at 265 nm and tetracyclines at 385 nm. Flow rate was 0.2 ml/min. Used gradient was:A:B 90:10 (0-35 min), 65:35 (35-36 min) and 90:10 (36-45/55 min). The temperature of the column oven was 40° C.

Method Validation

Blank milk samples (n = 7) were spiked with sulfonamides (SMR, STZ, SMX, SMTZ, SDOX, SCL and SDM) and tetracyclines (OTC, TC, DC, CTC) and chloramphenicol at the concentration of 200µg/kg each. Spiked and blank milk samples were handled as described before.

RESULTS

The majority of the collected milk samples were cow milk (78%); 10% goat milk, 4% a mixture of cow and goat milk and 8% not specified. Some samples were from one animal, some were pooled. Altogether, 480 raw milk samples were analyzed for antibiotic residues first with Delvotest® giving positive, negative or unclear result. Sixty two randomly selected positive and unclear samples were subjected to Trisensor screening. Finally, 10 Trisensor positive samples were analyzed with HPLC. Figure 1 specifies the screening process.

In Delvotest® screening, 24% of the analyzed samples were positive. Unclear and positive samples were altogether 39% when analyzed with Delvotest®. Sixty-two of the 185 samples requiring confirmation were tested with Trisensor. In all, 15 samples (3 %) showed positive results with Trisensor testing.

The screening test results by county and division are presented in Table 1. Except samples from one division, over 90% of the samples were analyzed. With Delvotest®, 30% of samples from Nandi and 16% of the samples from Makueni were positive. Spoilt milk samples are samples which were ranked as unusable or the result was neglected due to their appearance or smell during the Delvotest® testing.

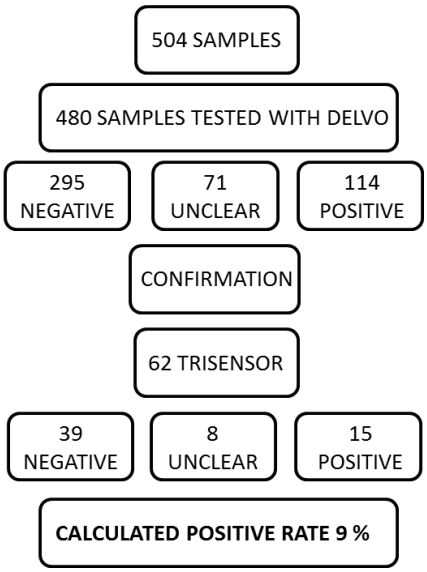

Figure 1. The course of screening procedure with results. Numbers refer to the number of samples tested and the number of results in each category.

Table 1. Number of screening tests and results of Delvotest®by county and division.

Number of samples		Makueni				Nandi			
		Total	Makindu	Kaiti	Wote	Total	Kaptumo	Kilibwoni	Laboret
Samples	504	247	77	104	66	257	94	65	98
Delvotest® ®	480	238	77	95	66	242	90	58	94
Negative	295	162	42	71	49	133	56	22	55
Positive	114	38	15	10	13	76	20	27	29
Unclear	71	38	20	14	4	33	14	9	10
Trisensor	62	42	15	13	14	20	4	12	4
Spoilt milk	60	15	6	8	1	45	29	1	15

Table 2. Screening test results by the Trisensor.

RESULT	Positive			Unclear
Antibiotic group	No. of Trisensor positives	% of Trisensor positive	% of all Delvo tested*	No. of unclear
	15		9	8
Beta-lactams	8	53	5	4
Tetracyclines	1	7	0.6	0
Sulfonamides	4	27	2.5	0
Beta-lactams, tetracyclines	2	13	0.4	3
Beta-lactams, tetracyclines, sulfonamides	0	0	0	1

* An estimation calculated from the portion of Trisensor tested samples in proportion to all Delvo tested (480).

Eight Trisensor positive samples were beta-lactam positive and represented cow milk samples from both Makueni and Nandi areas (Table 2). Four of the samples were sulfonamide positive: three unknown and one cow milk, all collected from Makueni area. One sample, a mixture of cow and goat milk, was tetracycline positive. Two samples were indicated as both beta-lactam and tetracycline positive; both cow milk.

Table 3. Validation results for blank milk spiked at a level of 200 µg/kg. SMX and STZ were eluted as a combined peak.

Validation	SMX/STZ	SMR	SCL	SDOX	SMTZ	SDM	OTC	TC	CTC	DC	CLF
Recovery (%)	103	103	96	80	61	47	115	29	52	61	102
SD (%)	26.0	6.1	7.9	8.9	10.4	18.4	6.2	13.3	6.9	4.4	33.4
LOD (µg/kg)	50	50	100	100	80	50	40	40	40	40	50

As footnote: SMX = sulfamethoxazole, STZ = sulfathiazole, SMX = sulfamethoxazole, SCL = sulfachloropyradizine, SDOX = sulfadoxine, SMTZ = sulfadimidine, SDM = sulfadimethoxine, OTC = oxytetracycline, TC = tetracycline, CTC = chlortetracycline, DC = doxycycline, CLF = chloramphenicol. SD = standard deviation, LOD = limit of detection.

None of the samples showed positivity for all the three antibiotics groups within the specific test range. Some of the samples were positive or unclear in several groups. Percentage is the result share of the corresponding positive or unclear group.

Ten samples of the Trisensor positives ones were analyzed with HPLC-UV. The analyzed samples showed to be negative for SMX, SMR, SCL, SDOX, SMTZ, SDM, OTC, TC, CTC, DC and CLF at and above the limit of detection (LOD) of the method. LODs and validation results are shown in Table 3.

Samples analyzed as beta-lactam positive with Trisensor were further analyzed for beta-lactams with LC-MSMS at Evira (Finnish Food Safety Authority, method accredited according to 17025 by Finas). All suspected beta-lactam positive samples were found to be negative for the analyzed beta-lactams (ampicillin, amoxycillin, benzylpenicillin, oxacillin, cloxacillin, and cephalexin).

DISCUSSION

Provided that all the 480 samples could have been tested with Trisensor, the number of Trisensor-positive samples would amount to 44 (9%). Provided that all the 480 samples would have been tested with Trisensor test, would the amount on beta-lactams by estimation be 5%, sulfonamides 2.5% and tetracyclines 0.6%.

The speculated proportion of positive samples, 9%, is similar with the latest study results from Kenya: 4% (Ekuttan et al., 2007) 16% (Kang'ethe et al., 2005) and 14% (Shitandi and Sternesjö, 2004). The mentioned results are conducted with microbial based screening tests, and might produce unspecific results in difficult sampling conditions. The identified antibiotic group shares are slightly different from the shares reported to be imported for food production animals: Mitema et al. (2001) found that tetracyclines are distinctly the largest group (61%), then sulfonamides (24%) and third largest group being beta-lactams (7%). Our findings suggest beta-lactams being most used (5%) at least in the study areas for dairy cattle, tetracyclines second (2.5%) and sulfonamides having a share of 0.4% of medicinal use.

On the other hand, recent findings from other East-African countries also indicate the absence of the residues from milk samples analyzed. Ngasala et al. (2015) analyzed 35 raw milk samples with Delvotest® and all were found to be negative for the antibiotic residues.

According to the results of this study, the prevalence of antibiotic residues is significantly higher in Kenya than in EU. In 2009, a total of 38 952 milk samples were tested for antibiotics in EU (excluding Germany) with screening methods. Only 0.13% were non-compliant samples as they contained antimicrobials. (European Commission staff working document, 2009).

Delvotest® is based on microbiological growth of the *B. stearothermophilus*. Delvotest® does not differentiate the reason for the positive result. The positive result can be caused either by antibiotic residues or by any other possible growth inhibitors, such as residues of detergents, other chemicals, competing micro flora or a big amount of somatic cells originating from mastitis. These growth inhibitors can end up in milk either by accident or on purpose. For example anthelmintics and other drugs including antibiotics (aminoglycosides, levamisole, oxyclozanide, benzimadazole and nitroxynil) are used against worm infections (Mitema et al., 2001, Keyyu et al., 2003) and could be one source of the growth inhibitors in the milk samples. Highly possible sources of errors in Delvotest® screening test beside the natural inhibitors in milk can also be the incubation type, human error and sample collection method (Kang and Kondo, 2001).

Milk sample spoilage by organoleptic evaluation was found high in our study: 12% (i.e. 60) of the samples were unusable and their results were therefore neglected. Declined samples were considered spoilt due to their appearance, consistency or smell. Thirty one percent of the 73 samples in one Nandi district, Kaptumo were spoilt. Seventy six percent of the samples that gave a positive or unclear result with Delvotest® were negative with Trisensor test. Trisensor test covers quite a range of beta-lactams, tetracyclines and sulfonamides (Table 4). There could be antibiotics in the samples not included in the list but one reason for unexplained inhibition of Delvotest® might be explained by the bad quality of the samples. There were breakdowns in the cold chain between collect-

Table 4. Test sensitivity for specific detectable compound compared with MRL (EU) and the detection limits (LOD) of the chemical confirmation methods.

	Delvotest®	Trisensor	HPLC-UV LOD	LC-MSMS LOD	MRL by EU
		µg/kg			
BETA-LACTAMS					
Penicillins					
Amoxycillin	3-5	3-5		0.5	4
Ampicillin	6-7	3-5		0.5	4
Benzylpenicillin/ Penicillin G	2-3	2-3		0.5	4
Cloxacillin	20-30	6-8		0.5	30
Dicloxacillin	10-20	6-8			30
Nafcillin	10	30-40			30
Oxacillin		12-18		0.5	30
Cefalosporins					
Cefacetrile		30-40			125
Cefalexin		2000		0.5	100
Cefalonium		3-5			20
Cefazolin		18-22			50
Cefapirin	6-8	6-8			60
Cefoperazone	3-4	3-4			50
Ceftiofur and metabolite	10-15	10-15			100
Cefquinome	20-30	30-35			20
TETRACYCLINES					**100**
Chlortetracycline		45-55	40		
Oxytetracycline		56-75	40		
Tetracycline		75-100	40		
Doxycycline		20-40	40		
SULFONAMIDES					**100**
Sulfacetamide		1000			
Sulfachloropyridazine		100	100		
Sulfadiazine	100-150	20-50			
Sulfadimethoxine		50	50		
Sulfadimidine/ Sulfamethazine	100-250	1-5	80		
Sulfadoxine			100		
Sulfamonomethoxine		30-50			
Sulfamethoxazole		800	50		
Sulfamethoxy-pyridazine		5-10			
Sulfamerazine		1-5	50		
Sulfapyridine		1			
Sulfaquinoxaline		50-100			
Sulfathiazole		50	50		

Detection levels for selected screening methods in µg/kg. Sources: Delvotest® instructions, Trisensor poster and EUR-Lex.

ion and analysis that could not be avoided. In this study milk sample collection methods were inconsistent.

Antibiotics degrade during storage especially when stored in ambient temperatures (Marth and Steele, 2001) and when the milk sample is even slightly spoiled. The beta-lactamase enzyme produced by the spoilage bacteria can destroy the beta-lactam residues (Guay et al., 1987). The same can occur with other antibiotic residues. This could be one reason for low residues detected with LC-MSMS by Evira after several months' storage of the samples. The milk samples included in this study were not analyzed for protein, fat, somatic cell or bacteriological contents. Furthermore, the HPLC and LC-MSMS analyses included a limited amount of different compounds of each antibiotic group and the compounds detected by Trisensor might not have belonged to the analyzed antibiotics.

Trisensor can react for example with dicloxacillin, nafcillin and several cefalosporins which were not included in the LC-MSMS analysis of beta-lactams (Table 4). The HPLC-UV method used was able to detect many of the same sulfonamides as Trisensor but the limit of detection of some compounds with HPLC-UV was higher compared to the ones of Trisensor. Delvotest® is reported to detect no tetracyclines and only sulfadiazine (SDZ) and sulfadimidine (sulfamethazine SMTZ) from the sulfonamide group and in a range around or above MRL (Table 4). Because Delvotest® was used for the first screening can it be assumed that the other

sulfonamides were eliminated and thus SDZ and SMTZ were the sulfonamides present in the sulfonamide positive samples detected by Trisensor test. Sulfadiazine is a quite common antibiotic in veterinary practice in many countries but the HPLC-UV method used in this study was unfortunately not able to detect SDZ because of disturbing impurities that in fact prevented the determination of some other sulfonamides too. Sulfonamides have their UV absorption on the low UV range (265 nm) where many impurities of biological origin can disturb the analysis.

The relative ease of access, availability and uncontrolled use of antibiotics make the choice of analytical techniques difficult and challenging in developing country conditions. All the available screening tests have limitations and specific compounds for detection within given range. As shown in Table 4, the coverage of the compounds and detection limits was not all-inclusive. This means that it is possible that the positive result given in a screening test was not confirmed later only because it wasn't included in the tested compounds. Availability of different beta-lactams only is wide and it is not known which antibiotics are specifically used in small dairy farms in Kenya.

CONCLUSIONS

This field study indicates that antibiotic residues are found still frequently in cow and goat milk produced on small-scale farms in Kenya. The results suggest that the farmers do not respect the recommended milk withdrawal times on dairy cows treated with antibiotics. Such practice calls for further training of farmers on the use of veterinary drugs. Again, the presence of any antibiotic residues in farm milk can increase public health risk among the dairy product consumers as milk is a staple food item in the Kenyan diet.

Because of the high prevalence of suspected false positive results and weak quality of the samples, microbiological inhibitor tests may not be best suited to the conditions and environment encountered in smallholder farms in Kenya. A screening test for antibiotics in raw milks should also include quality and microbiological testing to exclude possible samples with weak quality, especially if microbiological inhibitory tests are used as the main analyzing method. To get the most reliable situation of the use of antibiotics in developing countries, the cold chain for the sample storage should be immediate and continuous. Because of the relatively free markets and uncontrolled use of antibiotics the chemical confirmation should cover as many compounds as possible and be specific, preferably LC-MS.

ACKNOWLEDGEMENT

This study was carried out as part of the Safe Food Safe Dairy–project in Kenya. The project is supported financially by the Ministry for Foreign Affairs of Finland. The HPLC-UV multi-residue method for sulfonamide and tetracycline detection in milk was developed and modified in collaboration with the Finnish Food Safety Authority Evira, Finland.

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