INCIDENCES OF ANTIMICROBIAL RESIDUES IN RAW COW MILK FROM SMALLHOLDER FARMS IN UASIN-GISHU COUNTY.

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ABSTRACT

In a sustained effort to control and manage the acute proliferation of bovine mastitis, prolonged and overwhelming application of antimicrobial therapeutic agents has been deployed. The net effect of the increased use of these antimicrobials for a longer period translated to Antimicrobial Drug Residues (AMDR) in raw cow milk. The AMDR have been reported exponentially and globally. To address this hazard, the objective of this study was to determine qualitative and quantitative antimicrobial residues in raw milk from smallholder dairy farms in Moiben (0.8238° N, 35.3764° E) and Kapseret (0.4309° N, 35.2272° E) sub-counties of Uasin-Gishu. The study employed a prospective cohort design in which 216 cows on 81 smallholder farms were considered. A multi-stage sampling of pooled mid-stream milk for AMDR testing was analyzed using microbiological culture *Delvotest* and *HPLC-MS/MS* methods. In this study, the prevalence of AMDR in milk was 6.9%, with occurrence in Moiben at 4.5% and in Kapseret at 11.0%. Fifty-three percent (53.3%) of the samples that were positive for the three classes of antimicrobial drugs had AMDR concentrations which were above the Safe Maximum Residue Limits of >4.0 µg/ml, >3.1 µg/ml, and >0.2 µg/ml for Penicillin, Tetracycline, and Streptomycin, respectively. Maintaining and observing strict withdrawal periods and active and regular surveillance of antimicrobial residue levels in raw milk is highly recommended.

Keywords: Antimicrobials, bovine; mastitis, residue, and resistance

INTRODUCTION

One of the adverse public health effects of sustained efforts to manage bovine mastitis is the prolonged and overwhelming use of antimicrobial agents. These lead to the accumulation of Antimicrobial Drug Residues (AMDR) in raw milk used for human consumption (Worku *et al.*, 2017). The AMDR sometimes persist in milk beyond the recommended withdrawal period (Omwenga *et al.*, 2021). The AMDR in milk is a potential source of many health-related complications, including non-communicable diseases and food poisoning in humans when consumed. In Uasin-Gishu County, many adverse effects arise from excessive milk consumption containing antimicrobial drug residues. These complications ranged from rheumatoid heart diseases, hypertension, Antimicrobial Resistance (AMR) in humans and animals (Park *et al.*, 2016) and the development of bacteria-resistant genes to commonly used antimicrobial agents (Mohamed *et al*., 2020).

In the USA, the consumption of raw cow milk tremendously decreased because of the presence of high levels of antimicrobial residues (Welsh *et al.,* 2019). The presence of antimicrobial residues in milk between 26-60% exceeded the recommended federal maximum levels of 3 %, 37%, and 26% for Amoxicillin, Sulfamethazine, and Sulfathiazole, respectively (Welsh *et al*., 2019). Mohamed *et al*. (2020) reported an overall prevalence of antimicrobial residue at 24% and 30% in raw farm milk and 18% in raw milk from the markets in Benadir, Somalia. In Ghana, Addo *et al.* (2011) documented an overall antimicrobial residue prevalence of 3.1%, with prevalence in the central Ghana region at 5.3%, Volta region at 2.1% and Greater-Accra region at 3.3%. In North Eastern Ethiopia, antimicrobial residues in raw milk at the farm level were 23.0% (Worku *et al.*, 2017). In Kenya prevalence of 9.4%, antimicrobial residue in raw milk at the farm level and 5.7% in milk sampled from the markets were detected, and all these antimicrobial residues were above the European Union (E.U.), Safe Maximum Residue Levels (SMRL) as shown in Aboge *et*

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al. (2000).

A study in Azerbaijan – Iran reported that 26.% of raw milk samples collected from industrial dairies and 16% of raw milk from market collection centres showed detectible antimicrobial residues (Mohamed *et al*., 2020). Further, 30% of pasteurized milk samples in Azerbaijan produced positive *Delvotest* results for AMDR. In conclusion, this study recommended routine monitoring of antimicrobial residues in milk and dairy products due to their adverse public health side effects on humans (Mohamed *et al*., 2020)

In Korea, humans were the worst hit by the harmful effects of antimicrobial residues in milk. In order to mitigate this effect, the Food and Agriculture Organization (FAO), European Union (E.U.) and Korean Ministry of Food and Drug Safety set SMRLs at 0.2 µg/mL for Streptomycin, 3.1 µg/mL for Tetracycline and 4.0 µg/mL for Penicillin (Park *et al*., 2016). Chowdhury *et al*. (2015) in Chittagong, Bangladesh, while using these SMRLs, determined the mean concentrations of Amoxicillin in raw and commercial milk at 9.8 µg/ml and 56.2 µg/ml, respectively. In this study, Chowdhury reported levels significantly higher above SMRLs ($P \leq 0.05$) for Tetracycline, Ciprofloxacin and Amoxicillin residues; he further observed that the antimicrobial residues decreased in boiling raw milk.

In Uasin-Gishu, Kenya, the prevalence of mastitis among smallholder farmers was as high as the demand for locally-produced raw milk (Kosgey *et al*., 2018). The therapeutic utilization of antimicrobials for managing bovine mastitis was widespread on smallholder dairy farms in Uasin-Gishu, leading to increased AMRs in milk. Multidrug-resistant *Staphylococcus aureus,* one of the leading causes of bovine *Staphylococcal* mastitis in milk from smallholder farms (SHF), was two times higher than in larger farms (Kosgey *et al*., 2018). Most of the raw milk from the SHF was marketed locally and directly to the consumers and popular milk Automated Teller Machines (ATMs), respectively. The Kenya Government regulation of antimicrobial residue in milk, especially from SHF, is not currently well implemented. The large-scale farmers who export most of their milk adhere to the standards of the East African Code of Regulation for AMDR (Kosgey *et al*., 2018).

The AMDR contribute to huge micro and macro-economic implications associated with far-reaching consequences, for instance, unwarranted low milk quality, reduced milk quantity (Halasa *et al*., 2007), reduced profitability because of the high cost of antimicrobials and discarded milk due to high quantities of antimicrobial residues (Asli *et al*., 2017). This study aimed to determine the presence of AMDR in raw milk from smallholder dairy farms consumed by humans in the Moiben and Kapseret Sub-counties of Uasin-Gishu County. A High-Performance Liquid Chromatography-tandem Mass Spectrometry (HPLC-MS/MS) method for the quantitative determination of AMDR in raw milk was adopted from the study by Chowdhury *et al.* (2015). The method is sensitive and accurate for the determination of AMDR in milk. The method has been successfully applied to determine concentrations of Streptomycin, Cotrimoxazole, Kanamycin, Gentamycin, Tetracycline, Chloramphenicol, and Sulphamethoxazole. Also, the method has been employed to determine concentrations of four penicillins (*ampicillin, penicillin G, Amoxicillin* and *Penicillin V*) and their four major *beta*-lactamase enzymatic metabolites (*Ampilloic acid, Penilloic acid G, Amoxiilloic acid and Penilloic acid V*).

METHODS AND MATERIALS

Study area

The study was carried out in Moiben (0.8238° N, 35.3764° E) and Kapseret (0.4309° N, 35.2272° E) sub-counties in Uasin-Gishu County, the two sub-counties have similar weather patterns and conditions and were treated as one study area. The sub-counties exist in the same geographical zone on the western lower region of North Rift and the eastern side of the Lake Victoria basin. The rainfall in this study area is bimodal, ranging from 500 to 1500 mm, with an average temperature of 18 ^{o}C. In 2021, long rains were experienced between March and May. However, during the period for short rains (usually occurring in September-October), the two sub-counties experiences heavy downpour that starts in July extending to November 2021.

Study population

A total population of 211,020 dairy cows were considered for this study, of which 130,911 cows were in the Moiben

sub-County while 80,109 were in the Kapseret sub-County. Of these, 70% were exotic breeds, 20% were crossbreeds, and (10%) were indigenous. Each sub-County had five wards, and the cow population was distributed as follows: Karuna 53,732, Moiben 32,239, Tembelio 22,470, Sergoit 15,631, Kimumu 6,839, Kapseret 26,378, Ngeria 23,447, Kipkenyo 11,723, Langas 10,746 and Megun 7,816 cows. This information was as per the records of the County Directorate of Livestock production and County Integrated Development Plan (CIDP, 2013).

Study design and sample size determination

The study adopted a prospective cohort design where study cows were recruited at the farm level to determine the baseline results. All cows testing negative for mastitis were recruited into the study and followed up between January and October 2021 to determine the bovine mastitis endpoints.

The sample size was determined using Kasiulevičius formula on the determination of sample size in epidemiological studies (Kasiulevičius *et al*., 2006) as follows;

$$N = \frac{P_1(1-P_1) + P_2(1-P_2)}{} \times f(a,b)/ (P_2-P_1)^2 ; n = 0.613(1- 0.613) + 0.387(1-0.387) \times 10.5074/(0.387 - 0.613)^2$$

Where: n = sample size, f(a,b) = 10.5074 (at a = 0.05 (2 tail), b = 0.1), P_1 = prevalence effect in exposed population, P_2 = prevalence effect in non-exposed population, N = n + 10% attrition level = 98 + (10/100)×98×2 = 216.

Significance level = the cut-off below which to reject or fail to reject the null hypothesis P = 0.05 (5% margin of error and 95% confidence level).

A sample size of 216 lactating dairy cows was selected from 81 smallholder farms in Moiben and Kapseret.

Sampling design

The study employed a multi-stage sampling design comprising stratified, cluster and simple random sampling methods. Out of five sub-counties in Uasin-Gishu County, two sub-counties were randomly selected – Moiben and Kapseret. All the administrative wards and locations in each sub-County were purposively sampled. Farms with smallholder herds were chosen for the study with the help of local Animal Health Officers. The farmers milked the first few drops of milk and then collected the mid-stream milk into sterile sample vials (Bourabah *et al*., 2013). The Milk samples that were not processed immediately within eight hours were stored at (4-8) °C in the laboratory for one week or frozen at -20°C until they were ready to determine antimicrobial residues.

Qualitative determination of the presence of antimicrobial residues in milk

A microbiological Modified *Delvotest* method by Mohamed *et al.* (2020) and was adopted for screening antimicrobial residues in this study. This method is a broad-spectrum qualitative test for detecting AMDR in raw milk. The test is based on the growth inhibition of *Escherichia coli* (ATCC 25922). Living microorganisms produce waste products that result in (pH) changes in their environment. The colour change of the bromocresol blue pH indicator indicates the pH change. The AMDR will kill the *E. coli* (ATCC 25922) hence no change in pH. *E. coli* (ATCC 25922) produces acid and causes bromocresol purple to turn yellow. The AMDR prevents acid formation, and a purple or blue colour is observed. The minimum concentration of antimicrobials detected is 0.004 to 0.005 µg/ml.

Escherichia coli, preferably in Blood agar and MacConkey plates, were incubated at 37 ° C for 18-24 hours. A pure colony of the organism was picked and inoculated in a trypticase soy broth using a sterile wire loop and incubated at 37 °C for 18-24 hours. Using a sterile test tube and sterile droppers, equal volumes of the inoculum and milk sample were mixed. Approximately 2-3 drops of bromocresol blue indicator were added and mixed well using a vortex mixer and incubated at 37 °C for up to 4 hours. the colour change was monitored regularly at intervals of 1 hour. Positive results produced purple/blue colour while negative results produced yellow colour.

Quantitative determination of antimicrobial residue levels in milk

A High-Performance Liquid Chromatography-tandem Mass Spectrometry (HPLC-MS/MS) method for determination of antimicrobial residues in raw milk was adopted from a study by Chowdhury *et al*. (2015). The method was sensitive and accurate for determination of antimicrobial residue levels in milk. The method was successfully used to determine Streptomycin,

Cotrimoxazole, Tetracycline, Kanamycin, Gentamycin, Chloramphenicol, Sulfamethoxazole and the four types of Penicillin (Ampicillin, Penicillin G, Amoxicillin and Penicillin V) plus their major *beta*-lactamase enzymatic metabolites (Ampilloic acid, Penilloic acid G, Amoxiilloic acid and Penilloic acid V).

HPLC MS/MS procedure and sample extraction and cleanup

Raw milk samples were thoroughly homogenized. Custom standard solution mixtures with each chemical at 100 mg/ml concentrations stored at -20 ^{o}C in a freezer. The custom standard solution was kept at room temperature (20 – 25 ^{o}C) to thaw. The stock standard mixture of 1mg/ml per chemical was prepared by diluting with acetonitrile to 200 ml of custom standard in 20 ml volumetric flask. The prepared stock standard solution was then transferred into an amber-coloured vial with a teflon lined screw cap. Sample extraction and clean-up was adopted from the study by Chowdhury *et al*. (2015).

HPLC-MS/MS analysis

By operating Agilent 1200 LCMS/MS equipment using equipment's working instructions, the above extracts were consecutively subjected to calibration standards, quality control and matrix matched samples to qualitative and quantitative analysis as follows;

The extract vials were arranged in the autosampler and the batch developed sequentially starting with S0 for blanks followed by S1 - S5 calibration standards, QC1 quality control sample, test samples, QC2 quality control sample, and finally calibration standards S1 - S5, and the analysis method was loaded.

The weighted linear regression of the calibration curve used for each targeted chemical should be greater or equal to 0.95 ($r^2 \geq 0.95$) for it to be used for the analysis. Concentration of each chemical targeted from responses obtained from the calibration curve and scored in ug/ml were used and were acceptable (Chowdhury *et al.*, 2015).

Ethical approval

The study obtained ethical approval from Masinde Muliro University of Science and Technology, approval number (MMUST/IERC/155/2021) and approval from the Directorate of Veterinary Services Reference number (MOALF&I/SDL/DVS/GEN/VOL.1/57),

Statistical analysis

The data from the modified microbiological culture *Delvotest* and HPLC-MS/MS were coded and stored in a Microsoft Excel (Microsoft, USA) spreadsheet. The data were then exported into SPSS version 20 (Microsoft, USA) software and processed. The qualitative screening and quantitative determination of AMDR in milk were analyzed using descriptive statistics. Then Fisher's exact test was performed to determine the association between AMDR and mastitis status. The results were presented as percentages in the tables.

RESULTS

Qualitative presence of antimicrobial residues in milk

The overall prevalence of AMDR in milk used for human consumption on dairy farms in Moiben and Kapseret was 6.9%, with occurrence in Kapseret being higher than in Moiben (Table 1). Furthermore, the overall distribution of AMDR in all the wards in the present study area depicted sporadic trends and patterns. Karuna, Moiben, Tembelio, Kimumu, Kapseret, Ngeria and Langas wards reported positive AMDR results, while Sergoit, Kipkenyo and Megun wards had negative results (Table I).

Quantitative determination of antimicrobial residue levels in milk

Three classes of antimicrobial agents were identified, namely *Beta*-lactams (Penicillin), Tetracyclines (Tetracycline) and Aminoglycosides (Streptomycin). Of the seven samples detected with *Beta*-lactam residues, two (28.6%) had Penicillin levels (15.9 and 12.3 µg/ml, respectively) above SMRLs, two (50%) of the four samples detected with Tetracycline had drug residue levels (21.8 and 7.3 µg/ml respectively) above SMRLs. In contrast, all samples detected with Streptomycin residues had levels way above SMRLs (Table II).

TABLE I- QUALITATIVE ANTIMICROBIAL DRUG RESIDUES IN MILK

Sub-County	Ward	Number of cows n=216	Antimicrobial drug residues	
			Positive (%)	Negative (%)
Moiben	Karuna	55	3.6	96.4
	Moiben	33	3.0	97.0
	Tembelio	23	4.4	95.6
	Sergoit	16	0.0	100.0
	Kimumu	7	28.6	71.4
Total		134	4.5	95.5
Kapseret	Kapseret	27	14.8	85.2
	Ngeria	24	4.2	95.8
	Kipkenyo	12	0.0	100.0
	Langas	11	36.4	63.6
	Megun	8	0.0	100.0
Total		82	11.0	89.0
Grand Total		216	6.9	93.1

TABLE II- QUANTITATIVE PRESENCE OF ANTIMICROBIAL DRUG RESIDUES IN MILK

Class of Antimicrobial agent	Type of Antibiotics Residues	Occurrence of Antimicrobial Drug Residues in Sub-counties		Quantity of residues in samples (µg/ml)	SMRLs Standard controls (µg/ml)
		Moiben	Kapseret		
Beta-lactam	Penicillin	0	1	15.9	4.0
	Penicillin	1	0	12.3	4.0
	Penicillin	0	1	3.70	4.0
	Penicillin	0	1	2.80	4.0
	Penicillin	1	0	2.50	4.0
	Penicillin	0	1	2.40	4.0
	Penicillin	1	0	2.10	4.0
Tetracycline	Tetracycline	1	0	21.8	3.1
	Tetracycline	1	0	2.90	3.1
	Tetracycline	0	1	1.60	3.1
	Tetracycline	0	1	7.30	3.1
Aminoglycoside	Streptomycin	1	0	11.0	0.2
	Streptomycin	0	1	9.60	0.2
	Streptomycin	0	1	9.20	0.2
	Streptomycin	0	1	4.90	0.2
Total	-	6 (4.5%)	9 (11%)	-	-

SMRLs = Safe maximum residue levels

Correlation of antimicrobial residue with bovine mastitis

Overall, AMDR was detected and determined in 6.9% of cows, of which 1.0% was detected in mastitis-positive cows infected with gram-negative *Proteus vulgaris*. Most AMDR (93.3%) was determined in mastitis-negative cows. Penicillin was the predominant AMDR detected in cow milk at 46.6%, while Tetracycline and Streptomycin were detected at 26.7% each (Table III).

TABLE III- CORRELATION OF ANTIMICROBIAL DRUG RESIDUES WITH MASTITIS CULTURE RESULTS AND MICROBIAL PATHOGENS OF MASTITIS.

Parameter/variable	Cows in Sub-counties		Total cows in study area (%)	Antimicrobial residue		Fisher's exact test
	Moiben	Kapsaret		Positive (%)	Negative (%)	(P-value)
Mastitis culture results						
Positive	68	36	48.2	1.0	99.0	0.001
Negative	66	46	51.8	12.5	87.5	
Total	134	82	100.0	6.9	93.1	-
Microbial pathogens of mastitis						
Gram-positive	45	31	35.2	0.0	100.0	0.999
Gram-negative	23	5	13.0	1.3	98.7	
Total	68	36	48.2	0.5	47.7	
Type of antimicrobial residue detected						
Penicillin	3	4	3.2	46.6	-	-
Tetracycline	2	2	1.9	26.7	-	
Streptomycin	1	3	1.9	26.7	-	
Total	2.8%	4.2%	6.9%	6.9%	-	-

DISCUSSION

The overall prevalence of AMDR in raw milk used for human consumption on dairy farms in Moiben and Kapseret was 6.9%, with prevalence in Moiben at 4.5% and Kapseret at 11.0%. Mohamed *et al*. (2020) reported a higher prevalence of AMDR than in the current study. They obtained 26.0% AMDR in raw milk samples collected from local farms and 16.0% raw milk from market collection centres. In Kenya, Ouma *et al*. (2021) observed a high prevalence of antibiotic residues in raw milk purchased from shops and automated vendor machines at Juja (46.9%) and Githurai (26.1%).

Fifty-three percent (53.3%) of the samples that were positive for the three classes of antimicrobial drugs had residues concentrations above the Safe Maximum Residue Limits (SMRL), of Beta-lactams (Penicillin >4.0 µg/ml), Tetracyclines (Tetracycline >3.1 µg/ml) and Aminoglycosides (Streptomycin >0.2 µg/ml). In a similar study by Chowdhury et al. (2015) in Chittagong, Bangladesh, the mean concentration of AMDR in raw milk was 9.8 µg/ml, and raw commercial milk was 56.2 µg/ml for Amoxicillin. Tetracycline, Ciprofloxacin and Amoxicillin residues were at significantly higher levels above SMRL ($p \leq 0.05$). Different results where the presence of AMDR in milk was 26-60 % were reported by Welsh *et al*. (2019). In that study, the AMDR levels exceeded recommended federal limits for Amoxicillin by 3.0 %, Sulfamethazine by 37.0 % and Sulfathiazole by 26.0 %. The results in the present study were different because the utilization of Tetracycline, Penicillin and Streptomycin for treating bovine mastitis was more frequent and routine in Uasin-Gishu County (Ounah *et al*., 2021). Furthermore, the antimicrobials were less costly, affordable and readily available for use by farmers without Animal Health Officers' prescriptions. The presence of violative AMDR above SMRL in food products can cause health hazards to the consumer, including allergic reactions especially for penicillin and induce other severe pathological conditions such as cancers for sulfonamides and tetracyclines, anaphylactic shock and nephropathy for gentamicin and bone marrow toxicity for chloramphenicol (Priyanka *et al*. 2017). However, the main concern regarding exposure to AMDR in milk is the emergence of antibiotic-resistant strains of pathogens, complicating the treatment for both human and animal diseases (Arsène *et al*., 2022).

Overall, AMDR was detected in 6.9% of cows, of which 1.0% were mastitis culture-positive cows infected with

gram-negative *Proteus Vulgaris, w*hile 12.5% of AMDR was detected in cows free from mastitis. The variation of AMDR in association with mastitis status was significant (P<0.001). (Ounah *et al., 2022),* microbial pathogens associated with mastitis in Uasin-Gishu County showed increased antimicrobial resistance to Penicillin (80.8%), Tetracycline (51.9%) and Streptomycin (58.7%) as shown in Ounah (2022). Microbial resistance necessitated sustained and prolonged use of antimicrobial drugs for an effective cure, leading to the high drug residue levels observed in this study. Residue levels in milk above SMRLs have been associated with failure to observe prescribed milk withdrawal periods following the treatment of animals by farmers due to economic losses involved in discarding a large amount of milk in adherence to the withdrawal period (Priyanka *et al*., 2017).

CONCLUSION

Anti Microbial Drug Residues occurrence of 6.9% was observed in raw cow milk from Moiben and Kapseret sub-counties of Uasin Gishu. Three different antimicrobials were detected, namely Penicillin, Tetracycline and Streptomycin. Over 50% of the drug residue detected was above the Safe Maximum Residue Limit recommended in milk.

RECOMMENDATION

Maintaining and observing strict withdrawal periods, sustained and regular surveillance of antimicrobial residue levels in raw milk, and boiling raw milk to decrease AMDR levels before human consumption is highly recommended strategies to ensure adherence to safe public health standards.

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