Original Research Article

Risk Factors of Mastitis and Antibiotic Susceptibility of *Staphylococcus* spp. and *Escherichia coli* isolated from cows kept by farmers in Githunguri Sub-County, Kenya

ABSTRACT

Mastitis poses a major challenge in dairy cattle farming as it leads to production and economic losses. Some mastitis causing bacteria have shown resistance to some antibiotic and the risk factors associated with the antimicrobial resistance (AMR) are not well understood. The current study assessed the prevalence and risk factors associated with occurrence of mastitis in dairy cows kept by small-holder farmers in Githunguri Sub-County, Kenya. Further, susceptibility of the isolated *Staphylococcus* spp. and *Escherichia coli* to commonly used antibiotics was evaluated. The study design was cross sectional where 91 milk samples from 40 farms were sampled from two wards in the study area. Structured questionnaires were used to assess risk factors of mastitis. The milk obtained from lactating cows was first subjected to California mastitis test (CMT) and then taken to microbiology laboratory for isolation and identification of *Staphylococcus* spp. and *Escherichia coli* using standard bacteriological tests. Antibiotics sensitivity of the isolated bacteria was examined using disc diffusion method. Based on CMT, the overall prevalence of mastitis was 50.5%. The prevalence of mastitis was associated ($p<0.05$) with hygiene levels, being highest (69.2%) in the dairy cattle whose udder and leg hygiene were dirty. Highest prevalence of mastitis ($p<0.05$) was found in cows who were not milked regularly (52.4%) compared to those who were milked regularly (50%) ($p<0.05$). Cows which were cleaned using tap water had higher prevalence (53.3%) of mastitis compared to those cleaned using water from bore holes (45.2%) ($p<0.05$). Cows that produced between 21-30 liters of milk per day had the highest prevalence (66.7%) of mastitis, while those producing between over 30 liters milk per day had lower prevalence (25%) ($p<0.05$). The prevalence of *Staphylococcus* spp. and *E. coli* were 30.8% and 12.1%, respectively. *Staphylococcus* spp. isolates showed varied resistance to all the tested antibiotics with the highest resistance being against Oxytetracycline (57.7%) and Tetracycline (38.5%) but were sensitive to Vancomycin (100%), Ampicillin (100%), Chloramphenicol (96.2%), Penicillin (92.3%) and Gentamycin (92.3%). All the *Escherichia coli* isolates were sensitive to Chloramphenicol, Kanamycin, Gentamycin and

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Comment [1]: Important to say s aureus % not Staph app

Comment [2]: Staph aureus which are sensitive to penicillin are also sensitive to ampicillin and vice versa.Therefore the difference in % may be due to the different number of organisms being tested. Better mention as a proportion than a %

Comment [3]: Gentamicin check and correct spelling

Comment [4]: Spelling error

Oxytetracycline but were resistant to Ampicillin (88.9%) and Vancomycin (88.9%) amongst other antibiotics. In conclusion, half of the sampled cows had mastitis whose risk factors was associated with poor hygiene and irregular milking of the cows. The isolated bacteria showed varied resistance to commonly used antibiotics. To reduce the prevalence of the mastitis and AMR, there is need for extension service workers to train farmers on improved management of the mastitis.

Comment [5]:
E coli is inherently resistant to vancomycin and this should be 100%, should not even test for this and no guidelines to interpret.

Key words: Mastitis, prevalence, risk factor, antibiotic sensitivity, *Staphylococcus aureus*, *Escherichia coli*.

1. Introduction

Livestock production sector in Kenya contributes about 10% to Kenya's National Gross Domestic Product (GDP) and 42% to agricultural sector GDP [1]. In the country, Kiambu County is one of the leading milk producing areas and has several dairy processors [2]. In the county, the dairy sector is a source of income and employment to many people [3]. However, local extension officers in their reports have highlighted that mastitis pose a major problem in the dairy cattle industry where it causes production loss, increased cost of management, treatment and mortality in severe cases. However, the magnitude of the disease as well as risk factors have not been well studied.

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In other counties in Kenya, where dairy cows are kept, the prevalence of mastitis ranges from 44% to 89% [4,5,6,7,8]. In these studies, the highest prevalence of mastitis has been observed in cows which are aged, having higher parities, poor body condition, having previous udder infections and pendulous teats of the udder. Other risk factors for mastitis include use of poor milking techniques, inappropriate milking personnel or machine and poor housing [9,10]. Thus, the strategies which have been advocated for control of mastitis include improvement of udder hygiene, treatment of subclinical cases, observation of sanitary conditions like proper cleaning of milking utensils and culling of chronic affected cows [11].

In recent years, an increase in the incidence of mastitis in the dairy herds have led to over-use of antibiotics which have resulted in emergence and spread of antimicrobial resistance [12]. Other risk factors associated with emergence of AMR include misusing and underdosing of antibiotics [13,14]. According to a previous study [15], all (100%) isolates from milk of dairy goats from Thika Sub- County in Kenya were resistant to Penicillin. Further, recent studies in some counties

Comment [6]:
Isolates of what? Was this Staph aureus

in Kenya showed that bacteria isolated from milk of cows had high burden of resistance to common antibiotics [4]. Globally, this situation cuts across most of countries and thus bodies like FAO and WHO have called for increased AMR surveillance and studies to determine the risk factors associated with the phenomenon. The current study assessed prevalence of mastitis in dairy cows kept by small holder farmers in Githunguri Sub- County in Kiambu bacteria and thereafter determined the susceptibility profile of *Staphylococcus* spp. and *E. coli* to antibiotics commonly used in treatment of mastitis. The two bacteria were chosen as they have been reported to be highly prevalent in other studies in Kenya and cause severe mastitis [4].

Comment [7]:
The sentence does not make sense

2.0 MATERIALS AND METHODS

2.1 Study area

This study was carried out in Githunguri Sub- County, which is situated in Kiambu County between Latitude 0^0 55'0" S and Longitude 37^0 4' 60" E. It has an elevation of 1530 meters above sea level. The area receives a mean annual rainfall of 1380mm.The average temperature of the study area is 24.6 ℃ [16]. The sub-county covers an area of 173.50 square Kilometer and consists of five wards: Githunguri, Githiga, Ikinu, Komothai and Ngewa. It has a population of about 165,232 people. The livestock population numbers in the sub-county are 9,111 sheep, 57,008 cattle, 6,513 goats [17]. Small holder or small-scale dairy production system dominates in Githunguri area, whereby the exotic dairy cattle are mainly fed on home grown forage and commercial concentrates [18]. Most of the farmers in the area sell their milk through the Githunguri Farmers' Cooperative Society, which is the Kenya's third biggest dairy operation, producing 230,000 kilograms of milk per day [19,20].

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2.2 Study design and sampling strategy

A cross-sectional survey was conducted to determine the prevalence and risk factors of mastitis, and occurrence of AMR in dairy cattle. The sample size was determined based on the formula described in [21] for low animal populations. The population of lactating cows in the study area was less than 10,000. Using the formula, the sample size was 91 lactating cows. These animals were obtained from 40 households in Githunguri and Ngewa wards. The farms were randomly

Comment [8]:
Better to describe the paper here and give the reference at the end of the sentence.

Comment [9]:
On what basis were these 2 wards selected?

sampled from a list of farms supplied by the extension agent from Githunguri Farmers' Cooperative Society.

2.3 Milk sample collection and handling

Milk was collected aseptically from lactating cows as previously described previously [15]. California Mastitis Test (CMT) was done at the field level where a four-well plastic paddle was used. Foremilk was discarded, and then about 3ml milk was drawn into each well. An equal volume of CMT reagent was added, and gently agitated. After mixing of milk and the reagent, the result was read as 1 (negative), 2 (positive), 3 (positive) depending on the gel formation [22]. Thereafter, 10ml milk was collected into sterile universal bottles, labelled and then transported in cool box to JKUAT Medical Microbiology Laboratory for analysis.

Comment [10]: Give a reference for this method to show the sensitivity and specificity etc

2.4 Determination of presence of *Staphylococcus* spp. and *E. coli* in sampled milk

Culturing and identification of bacteria was done as previously described by (23, 24). The milk was cultured in Nutrient Agar (NA). The culture was incubated for 24 hours at 37^0C. Morphological and cultural characteristics were used to identify the bacteria using a light microscope. Sub-culturing was carried out using Mannitol Salt Agar (MSA) and Eosin Methylene Blue (EMB) to isolate *Staphylococcus* spp. and *E. coli* respectively. Bacterial identification of isolated bacteria was done based on colony morphological features and gram staining reactions on pure cultures and biochemical tests such as indole, catalase and oxidase tests.

Comment [11]: Why wasn't Staph aureus identified with at least coagulase test? Since this is a common virulent pathogen it is important to look at Staph aureus separately.

Comment [12]: What standard was used for antibiotic sensitivity testing?

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2.5 Questionnaire survey

Both open-ended and closed structured questionnaires were administered to the 40 farmers. The questionnaires composed of a series of questions which were administered to dairy farmers at farm level to determine the bio data, dairy herd size, mastitis related questions and the risk factors of mastitis including previous cases of mastitis in the farm, age, parity, body condition, and stage of lactation. The milk yield per cow was also included. Through direct observations, the udder and leg hygiene was categorized as; highly dirty, moderately dirty, slightly dirty and clean.

2.6 Data analysis

The collected data was entered into Ms Excel (Microsoft 2013, USA) and thereafter exported to a statistical package SPSS [Statistical Package for the Social Sciences, Microsoft 2013, USA) for the statistical analysis. A Chi-square test was used to evaluate relationship between risk factors and occurrence of sub-clinical mastitis ($p<0.05$).

3.0 RESULTS

3.1 Characteristics of farmers and cattle sampled

A total of 40 farmers from Githunguri and Ngewa wards of Githunguri Sub- County participated in the study. The demographic characteristics of the farmers and the sampled cattle are shown in Table 1. More women than men participated in the study and their occupation was majorly farming (85%). Majority (87.5%) of the sampled farmers had undergone formal of education. Exotic breeds were the predominant (66%) cattle breed while crosses accounted for 33.3%. The average number of cows per farm were five and were kept in farms of half acreage.

Comment [13]:
Formal education , no of in the middle

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Table 1: Characteristics of respondents in Githunguri Sub-County, Kenya

Factors	Categories	Frequency	Percentage
Gender	Male	18	45.0
	Female	22	55.0
Occupation of	Business	2	5.0
	Engineer	1	2.5
	Farming	34	85.0
	Pharmacist	1	2.5
	Teacher	1	2.5
	Veterinarian	1	2.5
Education	Primary	7	17.5
	Secondary	16	40.0
	Tertiary	12	30.0
	Informal	5	12.5

3.2 Prevalence of mastitis and identification of bacteria

California Mastitis Test was used to examine the prevalence of mastitis in the study area. The prevalence at cow level was 50.5% while the prevalence at udder level was 12.6%. Cows sampled from Githunguri Ward had a higher (29/50, 58%) prevalence compared to those from Ngewa Ward (17/41, 41.5%). The prevalence of *Staphylococcus* spp. and *E. coli* was 30.8% and 12.1%, respectively. The total number of *Staphylococcus* spp. and *E. coli* isolates were 29 and 11, respectively.

Comment [14]:
Not clear what this is?

3.3 Relationship between prevalence and risk factors of mastitis

The results of CMT were used to evaluate the relationship between risk factors and prevalence of mastitis. The prevalence of mastitis was significantly ($p<0.05$) associated with hygiene levels,

being highest (69.2%) in the cows whose udder and leg hygiene were assessed as dirty. The highest prevalence of mastitis was found in cows who were not milked regularly (52.4%) compared to those who were milked regularly (50%) ($p<0.05$). Cows whose udder were cleaned using tap water had higher prevalence (53.3%) of mastitis compared to those cleaned using borehole water (45.2%). Cows that produced between 21-30 liters of milk had significantly ($p<0.05$) higher prevalence (66.7%) of mastitis compared to other categories of production. Thus, udder and leg hygiene, milk yield, water source and frequency of milking were significantly ($p<0.05$) related to the prevalence of mastitis. However, the type of floor, frequency and use of teat dips and towels, culling of chronically infected mastitis cows, presence of drainage system, provision of bedding material, production system, removal of manure and cleaning of the house (cow shed) were not significantly related to the prevalence of mastitis ($p>0.05$).

Comment [15]: 52.4% and 50% is more than 100%. Also not much of a difference.??

Comment [16]: The table shows the other way round

Table 2. Relationship between risk factors and prevalence of mastitis in cows kept by small-holder farmers Githunguri Sub- County, Kenya (N=Number of sampled cattle)

Variable		N	Proportion positive	Prevalence (%)	P value
Age	1 year	10	0/10	0.0	0.500
	2-5 years	40	26/40	65	
	More than 5years	41	20/41	48.9	
Lactation stage	Early	15	6/15	40	0.794
	Mid	30	12/30	40	
	Late	46	28/46	60	
Parity	1-3	13	5/13	38.5	0.548
	4-7	42	18/42	42.9	
	7 and above	36	23/36	63.9	
Washing hands during milking	Before milking	40	19/40	47.5	0.162
	After milking	21	15/21	71.4	
	Before and after milking	30	12/30	40	

Comment [17]: Check the p values. They don't seem to be correct.

Comment [18]: If you analyse this as washing hands before milking or not there will be a significant difference. 1st and the last groups should be grouped together.

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Pendulous teats	Yes	56	29/56	51.8	0.587
	No	35	17/35	48.6	
Udder leg hygiene	Clean	15	8/15	53.3	0.027
	Slightly dirty	35	18/35	51.2	
	Moderately dirty	28	13/28	46.4	
	Highly dirty	13	7/13	53.8	
Milk yield	Less than 5 liters	10	4/10	40	0.018
	5-10 liters	30	13/30	43.3	
	11-20 liters	8	5/8	62.5	
	21-30 liters	30	20/30	66.7	
	31-40 liters	4	¼	25	
	41-50 liters	5	2/5	40	
	Above 50 liters	4	1/4	25	

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Type of Floor	Concrete	72	31/72	43.1	0.215
	Muddy soil	17	14/17	82.4	
	Both	2	½	50	
Use of teat dips and towel	Yes	45	20/45	44.456.5	0.975
	No	46	26/46		
Frequency of using teat dips and towels	Before milking	50	30/50	60	0.696
	After milking	30	14/30	46.7	
	Before and after Milking	11	2/11	18.2	
Frequency of milking	Twice	40	20/40	50	0.030
	Thrice	30	15/30	50	
	No regular schedule	21	11/21	52.4	
Water source	Borehole	60	32/60	53.3	0.015
	Tap	31	14/31	45.2	
Provision of beddings	Yes	51	30/51	58.8	0.305
	No	40	16/40	40	

Comment [19]: Each group had close to 50% prevalence. Check the p value.

Comment [20]: Text is not matching this data

Comment [21]: Check the p value? Send how it was calculated

Production system	Zero grazing	70	40/70	57.1	0.773
	Open grazing	21	6/21	28.9	
House cleaning	Yes	70	38/70	54.3	0.773
	No	21	8/21	38.1	
Removal of manure	Daily	68	29/68	42.6	0.830
	Weekly	23	17/23	73.9	
Drainage System	Yes	66	26/66	39.4	0.108
	No	25	20/25	80	
Culling of chronic mastitis cows	Yes	20	5/20	25	0.484
	No	71	41/71	57.7	

Comment [22]: The prevelance is markedly different . Check the p value.

3.5 Antibiotic sensitivity

Antibiotic sensitivity was tested against the isolated *Staphylococcus* spp. and *E. coli* (Table 3). *Staphylococcus* spp. isolates showed varied resistance to all the tested antibiotics with the highest resistance being against Oxytetracycline (57.7%), Tetracycline (38.5%), and Kanamycin (23.1%). Nevertheless, majority of the *S. aureus* were highly sensitive to Vancomycin, (100%), Ampicillin (100%), Chloramphenicol (96.2%), Penicillin (92.3%) and Gentamycin (92.3%). *E. coli* were shown to be more sensitive to Chloramphenicol (100%), Kanamycin (100%), Oxytetracycline (100%), Gentamycin (100%), Oxytetracycline (100%), Tetracycline (88.9%) Streptomycin (88.9%), and Penicillin G (77.8 %) while being highly resistant against Ampicillin (88.9%) and Vancomycin (88.9%).

Comment [23]: Very unusual. How was this interpreted? What standard?

Comment [24]: Spelling

Comment [25]: Spelling

Comment [26]: Impossible. There is some issue in the method.

Comment [27]: This should be 100% as inherently resistant

Table 3: Antibiotic susceptibility patterns of bacterial isolates from milk of cows kept by farmers in Githunguri Sub- County, Kenya

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Antibiotics	Resistant *Staphylococcus* spp. (%)	Intermediate Resistant *Staphylococcus* spp. (%)	Susceptible *Staphylococcus* spp. (%)	Resistance *E. coli* (%)	Intermediate *E. coli* (%)	Susceptible *E. coli* (%)
Ampicillin	0(0%)	0(0%)	26 (100%)	8 (88.9%)	1 (11.1%)	
Chloramphenicol	0(0%)	1(3.8%)	25(96.2%)	0 (0%)	0 (0%)	
Kanamycin	6(23.1%)	1 (3.8%)	19 (73.1%)	0 (0%)	0 (0%)	9(100%)
Penicillin G	0(0%)	2 (7.7%)	24(92.3%)	2(22.2%)	0 (0%)	7(77.8%)
Gentamycin	0 (0%)	2(7.7%)	24(92.3%)	0 (0%)	0 (0%)	9(100%)
Streptomycin	3(11.5%)	2(7.7%)	21 (80.8%)	0(0%)	1(0%)	8 (88.9%)
Oxytetracycline	15(57.7%)	1(3.8. %)	10(38.5%)	0(0%)	0(0%)	9 (100%)
Vancomycin	0 (0%)	0(0%)	26(100%)	8 (88.9%)	1(11.1%)	0
Tetracycline	10(38.5%)	6 (23.1%)	10(38.5%)	1 (11.1%)	0 (0%)	8(88.9%)

Comment [28]: Very unusual. In humans 90% resistance to penicillin and ampicillin in Staph. What was the standard used to interpret the results?

Comment [29]: Not possible. No guideline to interpret this.???

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4.0 DISCUSSION

The current study is a continuation of the ongoing studies investigating the occurrence of mastitis and AMR in milk of dairy cows kept by smallholder farmers in Kenya. In the present study, the overall prevalence of mastitis was close to that reported in Rwanda (51.8%) [25] but was lower compared to that observed in cows from other parts of Kenya (64-87.4%) [26], Ethiopia 74.7% [27] and Uganda 86.2% [28]. However, this study showed a higher prevalence of mastitis compared to those done in some parts of Kenya (36% -37%) [29,30] and Zimbabwe (21.1%) [31]. The variations in prevalence in these studies could be attributed to differences in management, environmental, epidemiological and breed factors.

Comment [30]: Discuss the sensitivity and specificity of the test used to diagnose mastitis. Could these organisms be contamination with colonising flora?

In our study the burden of mastitis was found more common in cows kept and milked under poor hygienic conditions. Poor udder and leg hygiene often leads to higher microbial loads leading to occurrence of mastitis [32]. The higher prevalence of mastitis in cows where borehole water in cleaning cow's udder could be because of water contamination by environmental bacteria such

as *E. coli* [33]. The current study showed that mastitis cases were higher in late lactation stage compared to other stages and this could be attributed to prolonged exposure during the protracted lactation period [6]. As reported by others [34], cows with higher parities were associated with increased prevalence of mastitis. In our study, the peak in prevalence of mastitis was observed in cows having 21-30 litres but thereafter declined. Some studies have showed that occurrence of mastitis increases as milk yield increases [35]. In the current study, the decline in prevalence in cows having over 30 litres of milk could be due to improved management by farmers whose cows produced this amount of milk.

In our study, both *Staphylococcus* spp. and *E. coli* were isolated from the milk. However, it should be noted that our study focused on isolation of these two bacteria and there could have been other species of bacteria in the milk. *Staphylococcus aureus* is a contagious bacterium which can spread from one cow to the other through contact during unhygienic milking procedures observed in the current study. On the other hand, *E. coli* infections could be due to fecal contamination on the udder and teats [36]. *Escherichia coli* is a ubiquitous organism found in the environment surrounding dairy cow, such as bedding and borehole water that was commonly used for cleaning the udder in the study area. The bacterium invades the udder through teat, proliferates and initiates inflammatory response in dairy cows leading to mastitis [33].

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The bacteria that were isolated from the milk samples were generally mostly sensitive to Gentamicin and Chloramphenicol. However, *E. coli* and *Staphylococcus* spp. showed high resistance to Vancomycin and Oxytetracycline, respectively. Similar profiles of resistance have been reported elsewhere in Uganda [37], Kenya [4,38] and Tanzania [39]. The susceptibility of *Staphylococcus* spp. and *E. coli* to Chloramphenicol and Gentamicin could be due to their infrequent use in mastitis treatment as most farmers in the study area use Penicillin (Veterinary officer, Guthunguri Sub-County personal communication). The resistance of bacteria to Penicillin may be attributed to production of beta-lactamase enzyme that inactivates Penicillin and other related antibiotics [40]. According to studies done in Tanzania [41] and Uganda [42], *S. aureus* showed high resistance to Oxytetracycline and Tetracycline. In the current study, resistance of *Staphylococcus* spp. to Oxytetracycline and Tetracycline accounted for most of the cases which is similar to a study undertaken in Uganda [37].

Comment [31]:
E coli is an indication of faecal contamination than environmental

Comment [32]:
In this study you have not identified Staph aureus???

Comment [33]:
E coli is inherently resistant to vancomycin and it is nothing to discuss about

Comment [34]:
Names of antibiotics need not start with a capital letter

Further, the present study showed that some *Staphylococcus* spp. isolates exhibited multidrug resistance pattern especially to Oxytetracycline and Tetracycline. *Escherichia coli* isolates exhibited resistance pattern to Ampicillin and Vancomycin which is similar to findings of previous studies[43, 44]. This could be attributed to the erratic and extensive use of antibacterial drugs without prior antimicrobial susceptibility testing.

Comment [35]:
Vancomycin is not acquired resistance in E coli

5.0 CONCLUSIONS

Half of the sampled cows in the current study had mastitis whose risk factors was associated with poor hygiene and irregular milking of the cows. The bacteria isolated from the milk included *Staphylococcus* spp. and *E. coli*. To stem the occurrence of the mastitis, extension service workers should train farmers on improved management of the disease. *Staphylococcus* spp. isolates showed varied resistance to all the tested antibiotics with the highest resistance being against Oxytetracycline, Tetracycline and Kanamycin. *Escherichia coli* were shown to be highly resistant against Ampicillin and Vancomycin. Local veterinarians should consider using prudent measures to manage the occurrence of AMR especially on using the antibiotics which were shown to be active against the isolated bacteria.

Comment [36]:
Don't mention this

Comment [37]:
What antibiotics are recommended ? Not clear

UNDER PEER

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