UNIVERSITY OF NAIROBI

COLLEGE OF AGRICULTURE AND VETERINARY SCIENCES

FACULTY OF VETERINARY MEDICINE

JVM 561: RESEARCH PROJECT

STUDY ON THE POSSIBLE CAUSES OF NEGATIVITY OF SUSPECT MASTITIC MILK SAMPLES UPON CULTURE

BY

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J30/2017/2010

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DECLARATION

This project is my original work and has not been presented for a degree in any other university.

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SIGN _____ DATE 20th April 2015

This project report has been submitted for examination with my approval as the University supervisor.

PROFESSOR SAMUEL M. ARIMI

SIGN _____ DATE 20 April 2015

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CHAPTER 1

1.0 ABSTRACT

Mastitis is a problem in dairy herds with significant economic importance due to the losses accompanying it. Management of this problem depends on the identification of the causative agent and use of appropriate antibiotics to manage the infection.

A study was conducted from October 2014 to January 2015 in Kabete Veterinary Laboratories to investigate the various causes of negativity of cultures from suspect mastitic milk samples in the laboratory.

A retrospective survey was conducted by going through the laboratory's records from January 2012 to October 2014 to determine the types of bacterial isolates in bovine milk samples during this period. Twenty fourmilk samples were analyzed for the presence of antibiotic residues and fungal organisms.Inhibitory substances test was done usingthe agar diffusion method.Questionnaires wereadministered to clients bringing suspect mastitic milk samples to the laboratory to investigate the handling of the samples.

It was discovered that most farmers collected the samples without proper disinfection of the teats in unsterile containers and then delivered to the laboratory unfrozen. Also, most farmers had treated their animals before receiving culture results. Most of the samples analyzed contained antibacterial residues but still showed growth after culture. It was suspected that this was due to antimicrobial resistance by the mastitis causing organisms.

CHAPTER 2

2.0 INTRODUCTION

Mastitis refers to inflammation of the mammary gland. It is the most common problem in dairy cattle and is of significant economic importance. Culture and identification of the causative agents is essential for effective treatmentand control of the infection. However, laboratories sometimes report no growth upon culture of the samples while the cows are still showing clinical signs of mastitis. Presence of inhibitory substances like antibiotics and disinfectants in milk samples(Maurice, 1984; Larry, 2001) is one of the causes of this problem. Also the milk sample could contain other organisms not routinely cultured for (Gonzalez, 1996)hence report of negative culture. This study was meant to investigate various causes of negative cultures in our laboratories.

The purpose of culturing mastitic milk samples is to identify the causative agent for effective treatment and control of the infection. False negative cultures are a disadvantage for the farmer as they may assume their treatment regime has worked while in fact it has not.This is because these cows have been observed to still show clinical signs of mastitis for certain periods of time. Knowing why the suspect mastitic milk samples are negative upon culture helps in determining the course of action to be taken in managing mastitis cases.

CHAPTER 3

3.0 LITERATURE REVIEW

The MinnesotaDairy herd improvement association (www.mndia.org; 25/10/2014) has postulated some of the reasons for getting negative culture results in milk samples from cows with mastitis and high somatic cell counts.These include traces of antibiotics and disinfectants in the milk that inhibit growth or kill the pathogens. Improper handling, collection of the sample or transport delay may reduce the number of mastitis causing bacteria to non-detectable levels or allow other contaminating microorganisms to overgrow.

The pathogen at time of collection may be below detectable levels (10- 100 organisms/ml) particularly for *Staphylococcus aureus*, coliforms and Mycoplasma(Richard, 2008).The organism itself may not be viable and clinical signs maybe due to bacterial products like toxins. This may be the case after treatment or when the pathogen has been killed by the immune system but the gland has not recovered fully hence showing high somatic cell counts. Also, the organism may not be grown by routine culture (fastidious organisms) and may require special nutrients e.g. anaerobes or may require more time to grow e.g. fungi. Sampling cows too soon after treatment or milking when bacterial counts are low may also result in no growth upon culture.

Culturing of milk samples from cows with high somatic cell counts and abnormal milk to get the causative agent is important for making informed decisions on treatment and prevention of the infection and also establishing a milking order in the herd so that cows with infectious conditions like *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma spp.* are milked last or in a separate unit to limit the spread of the infection.

This information is also useful when doing dry cow therapy to compare somatic cell counts before drying and at beginning of lactation to assess the response to treatment.Culling cows with chronically high somatic cell counts and positive cultures of *Mycoplasma spp.* may benecessary to control the spread of infection. Culture and identification of causative agents of mastitis is also important in vaccination protocols e.g. when using core antigen vaccines for coliform mastitis.

On farm milk culturing (Sterner, 2007; Hess, 2003) has been used to minimize time used to process samples in laboratories hence timely treatment and assessment of response as the person culturing is also responsible for treatment. It also identifies negative cultures where the animal's immune system has responded to the infection. In this protocol, cultures are classified as Gram negative, Gram positive, and no growth. The benefit of on farm culturing is getting maximum treatment success while reducing the amount of antibiotics used. This helps reduce the risk of antibiotic resistance which is a problem in dairy herds.

Fungal mastitis (Gonzalez, 1996) has also been suspected in cultures that are negative after 24 hours of incubation. This is considered in cows that are not responsive to treatment or those showing intensification of clinical signs of mastitis after intramammary infusion of antibiotics.Fungal mastitis is attributed to treatment being directed to other pathogens and using contaminated syringes and canulas or contaminated antibiotic preparations.

Candida albicans and *Cryptococcus neoformans* are some of the organisms isolated. *Candida albicans* grows on blood agar at 37°C for 24-48 hours (Radostits, 2000). The colonies are opaque, white or yellowish, and smooth at first. Their texture is creamy or pasty, and in a

microscopic smear consists of oval to round budding blastospores. *Cryptococcus neoformans* also grows well on blood agar at 37°C forming colonies within 48-72 hours. Colonies initially are pale and pasty, becoming honey-brown and mucoid later(Gonzalez, 1996).

CHAPTER 4

4.0 OBJECTIVE

This study was meant to investigate how samples are handled before reaching the laboratory, analyze milk samples showing no growth for antibiotic residues and also investigate other organisms i.e.fungias a cause of mastitis.

4.1 Hypothesis

Presence of antibiotic residues and organisms other than common mastitis causing bacteria in milk samples is a cause of negative cultures in laboratories.

CHAPTER 5

5.0 METHODOLOGY

5.1 Study area

This study was carried out at Kabete Veterinary Laboratories and included milk samples collected from January 2012 to December 2014. Both quantitative and qualitative methods were applied in the data collection.

The samples obtained from the laboratory were mainly from Nairobi and Kiambu counties. They were refrigerated at 4^0C and delivered to the department of Public Health Pharmacology and Toxicology, College of Agriculture and Veterinary Sciences in a cool box.

5.2 Study methods

First, a retrospective survey was conducted by going through the laboratory's records from January 2012 to October 2014 to determine the types of bacterial isolates in bovine milk samples during this period. Inquiries were also made on further tests done to these samples.

Second, twenty fourmilk samples were obtained and analyzed for antibiotic residues and fungal organisms at the PHPT laboratory, University of Nairobi. These were selected purposefully during the period of data collection from those animals showing clinical signs of mastitis.

The media used for the analysis were Blood agar (Oxoid), constituted by mixing 40g of the media base in distilled water, and Mueller Hinton agar (Oxoid), constituted by mixing 38g of the base in distilled water. The media were sterilized and poured into sterile Petri dishes.

The milk samples were streaked on blood agarand incubated at 33^0C for 24 – 72 hours to see any fungal growth present. Fungal isolation is best done at lower temperature than 37^0C. (Radostits, 2000)

To check for inhibitory substances, the samples were incubated on Mueller Hinton agar plates containing a sensitive bacteria (*Micrococcus luteus*) using the agar diffusion method. *Micrococcus luteus* was first sub-cultured and transferred to a vial of distilled water which was then swabbed over the Mueller Hinton agar plates.Four wells of 8mm diameter were then bored in each agar plate using a sterilized metal tube. Using a micro titer pipette, 100µl of each sample put in a labeled well. The plates were allowed to dry and then incubated at 37^0Cfor 24 hours. The inhibition zone caused by inhibitory substances was measured and recorded for each sample. The diameter of the well was included in measuring the inhibition zone.

Finally a questionnaire[Appendix 1] was administered to clients bringing mastitic milk samples to the laboratory to investigate the collection method of the sample, time taken for transport, number of animals in the herd and those showing clinical signs of mastitis, any treatment given and the response.

CHAPTER 6

6.0 RESULTS

6.1 Retrospective Study on the Bacterial Isolates at Kabete Veterinay Laboratory

Findings of the retrospective surveyconducted by going through the laboratory's records from January 2012 to October 2014 to investigate incidences of negative cultures and common isolates from positive samples are as presented in figure 1 below.

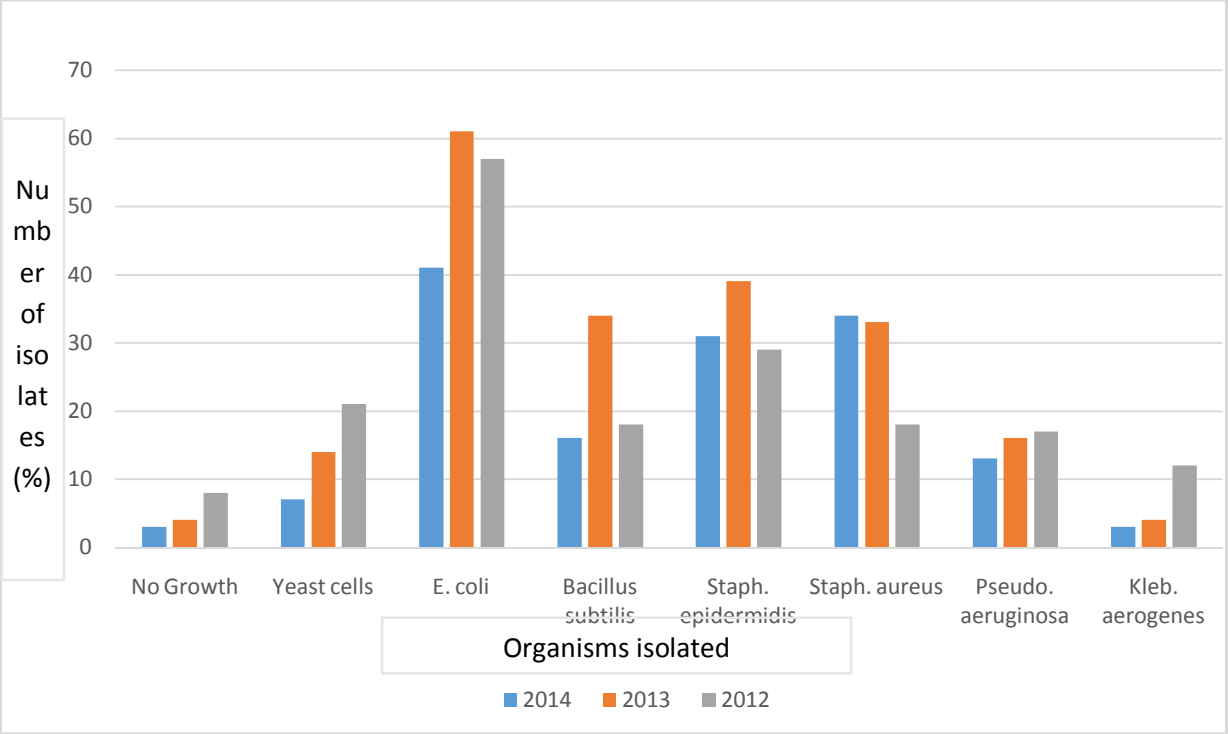

Figure 1: Type and number of bacterial and yeast isolates for the period January 2012 to October 2014

It was observed that *Escherichia coli* was the most isolated bacteria. There were also incidences of no growth cultures and fungal mastitis though not in large numbers.*Bacillus subtilis, Staphylococcus epidermidis, Staphylococcus aureus* and *Pseudomonas aeruginosa* were also isolated from many milk samples. Other organisms isolated in the laboratory were *Klebsiella aerogenes*, and *Proteus mirabilis*.

6.2 Results of the questionnaires

Clients bringing mastitic milk samples to Kabete Veterinary Laboratory filled the questionnaire shown in Appendix 1 with details on the collection method of the sample, time taken for transport, number of animals in the herd and those showing clinical signs of mastitis, any treatment given and the response.

The results of the 18 analyzed questionnaires were tabulated as shown in Table 1 below.

Table 1: Questionnaire results on sample handling and treatmentof animals

Test question	No. of clients/ 18
unfrozen samples	17
had suffered from mastitis before	11
collected sample by themselves	15
collected sample without teat disinfection	10
sample collected after milking	12
used unsterilized containers	10
animal treated before culture	12

It was observed that most clients (83%) collected the samples by themselves and delivered them unfrozen to the laboratory. Farm assistants were also reported to have collected the samplesin some instances.Some clients(55%) collected the samples without proper disinfection of the teats, some collected the sample after milking (67%) and 55% in unsterile containers. A large number of clients (60%) reported that their animals had suffered from mastitis before. Some of the clients (67%) reported to have treated their animals before culture of the milk samples and that the treatment was recommended by a veterinarian. They also reported that no marked improvement was achieved after the treatment.

6.3 Activities in the Laboratory

There was an interest to find out routine activities in Kabete Veterinary Laboratory after receipt of the samples. The laboratory mainly used Blood agar to culture the samples which were incubated at 37^0C for 24 hours. The sample was also sub-cultured in glucose broth to amplify the organisms in case there was no growth on Blood agar in the first 24 hours. For suspect fungal organisms, Sabouraud's dextrose agar and Maltose agar were used. Clients who were found to have treated their animals less than 5 days prior to collection of the samples were asked to collect the sample two weeks after antibiotic levels had decreased.

Also, the laboratory did sensitivity testing on the isolated organisms using antimicrobial disks containing Ampicillin 25mg, Tetracycline 100mg, Nitrofuran 200mg, Nalidixic acid 30mg, Streptomycin 25mg, Sulphamethoxazole 200mg, Cotrimazole 25mg and Gentamicin 10mg. The client was then advised to change treatment accordingly.

Identification of the cultured organisms was done by observing cultural morphology, microscopic characteristics and biochemical tests to get the species level.

A report was then made and presented to the client. This usually took about 2-4 days.

6.4 Results on inhibitory substances test

Figure 2 shows the results of inhibitory substance tests done on 24 samples. The control used was 50mg of Tetracyclin 20% (Norbrook). Diameter of the culture well (8mm) was included when measuring diameter of inhibition. A diameter of more than 9mm was considered to have shown inhibition.

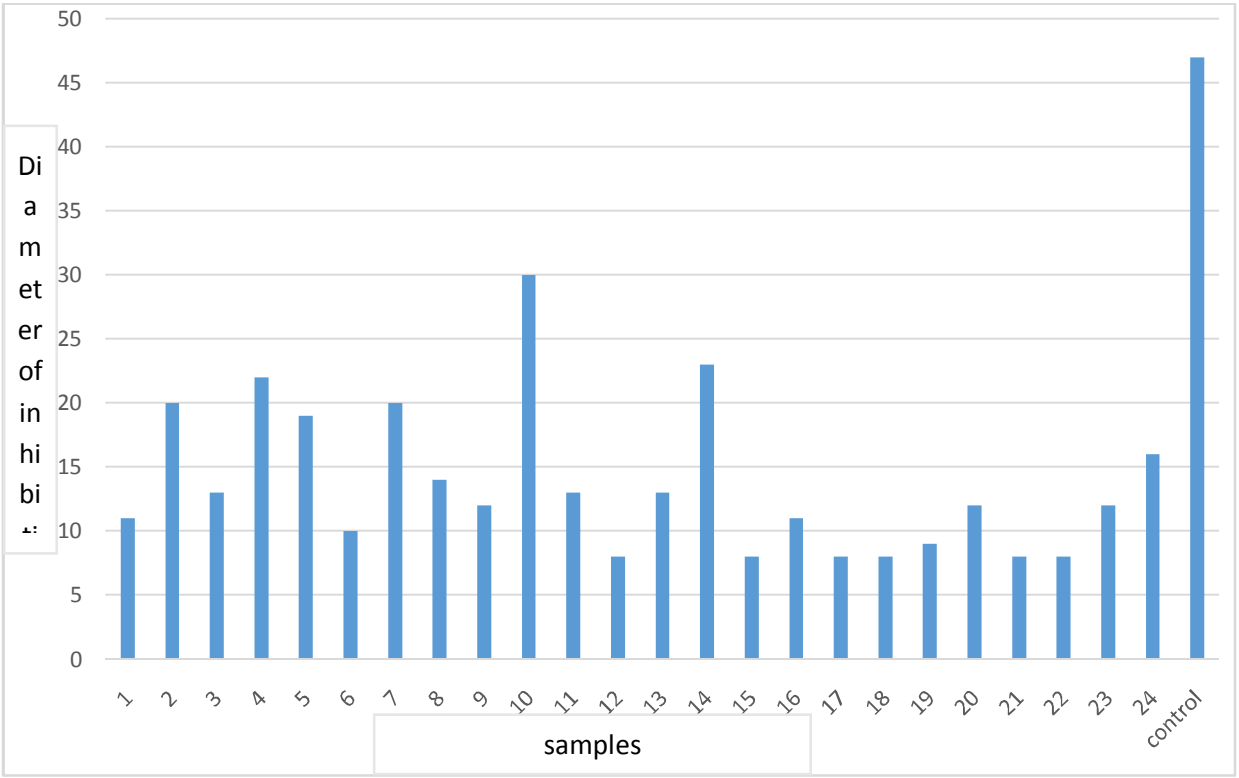

Figure 2: Results of inhibitory substances testing by agar diffusion method

Just as farmers reported to have treated their animals before collection of the samples, it was observed that most of the milk samples (75%) had antibiotic residues as shown in figure 2 above. Samples 12, 15, 17, 18, 21 and 22 had no area of inhibition on the Mueller Hinton agar plates. However, all samples showed bacterial growth in the first 24 hours of culture.

CHAPTER 7

7.0 DISCUSSION

It was observed that *Escherichia coli* was the most commonly isolated bacteria at Kabete Veterinary Laboratories. This was suspected to be attributed to the fact that most clients collected the samples by themselves and delivered them unfrozen to the laboratory. Some also collected the samples without proper disinfection of the teats, some collected the sample after milking and some in unsterile containers. These conditions could have led to contamination of the samples with environmental contaminants like *E. coli*.This displays lack of knowledge by the farmers with regard to hygiene and methods of sample collection for meaningful results. This could be due to inadequate guidance by extension personnel.

It was observed that the culture isolation trends remained fairly constant in the three years investigated, hence it was suspected that the farmers had not put in place concrete control measures for these mastitis causing organisms. It was concluded therefore that the farmers

were mostly concerned with treating the presenting infection and less with the elimination and control of the infection.

The laboratory processed samples from animals whose antimicrobial levels after treating had been allowed to decrease. This prevented the direct action of high antimicrobial compounds in the system from being seen.

Many samples analyzed (75%) contained inhibitory substances as shown in graph2. However, this did not prevent growth of bacteria in the first 24 hours of culture, an observation not consistent with the expected results. This was suspected to be due to the fact that bacteria develop antimicrobial resistance when they are constantly exposed to small doses of antibiotics. Also, it was suspected that the concentration of these antibiotic residues may be below the therapeutic levels hence with less killing action.

In order to see the direct effect of antimicrobial substances in milk samples, it would have been better taking samples directly from the infected animals using standard procedure to avoid contamination. However, this was not possible in this student research setting due to limited time and resources.

CHAPTER 8

8.0 CONCLUSIONS

Farmers have limited knowledge on management and control of mastitis in dairy cattle. They were mostly concerned with treating the presenting infection and less with the elimination and control of the infection.

Samples collected without following proper procedure may get contaminated with other microorganisms hence wrong medication recommended for treatment. Further treatment of these animals without taking samples for testing warrants negative results and also development of antimicrobial resistance hence interfering with treatment of mastitis.

Veterinarians sometimes recommend treatment to farmers before being certain of the causative agent of mastitis hence adding to the problem of antibiotic resistance.

The results obtained in this study show that there is a long way to go in reducing the incidences and eventually controlling mastitis in the dairy industry in Kenya. Rigorous education of farmers with regard to mastitis is hence called for.

CHAPTER 9

9.0 RECOMMENDATIONS

Based on the findings of this research, some of the recommendations made were;

i. That farmers be trained or advised on methods of collection and preservation of samples.

ii. That farmers should be advised not to treat animals before getting culture results to minimize the risk of antibacterial resistance.

iii. Veterinarians should use antibiotics judiciously to avoid antimicrobial resistance.

iv. Veterinarians and farmers should adopt on-farm culturing as described by Sterner 2007, in order to identify and treat the specific cause of mastitis promptly.

v. Where negative cultures are observed in the laboratory, cultures for other organisms e.g. fungi should be done before declaring the sample sterile.

vi. Treatment of mastitic animals should be done after culture and sensitivity testing of milk samples where possible, to increase efficiency of treatment. If not possible, the treatment regime should be changed to the most sensitive as soon as culture and sensitivity results are obtained.

CHAPTER 10

10.0 REFERENCES

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CHAPTER 11

11.0 APPENDICES

Appendix 1: Questionnaire for clients

UNIVERSITY OF NAIROBI

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JVM 561: RESEARCH PROJECT

I am doing a research on the possible causes of negativity of suspect mastitic milk samples upon culture in the laboratory.

I would appreciate if you took some time to fill this questionnaire with details on the milk sample you delivered to this laboratory.

Please tick in the boxes where necessary.

Q1) Are you the owner of the animals or the veterinarian? _____
Other (specify) _____

Q2) When was the sample collected? _____

Q3) Method of transportation: In cool box ☐ In bottle unfrozen ☐

Q4) Has the cow suffered from mastitis before? Yes ☐ No ☐

Q5) If yes, how many times has she had the disease? _____

Q6) Age of the cow: _____ Parity (Number of times she has given birth) _____

Q7) Did you collect the sample yourself? Yes ☐ No ☐

Q8) If no, who collected the sample? _____

Q9) How was the sample collected? (*fill both part A and B*)

A) i) Straight from affected quarter after teat disinfection ☐

ii) Straight from affected quarter without teat disinfection ☐

B) i) Before milking the cow ☐

ii) After milking the cow ☐

Q10) What type of container was used to collect the sample?

A) i) Glass sterilized ☐ B) i) Plastic sterilized ☐

ii) Glass unsterilized ☐ ii) Plastic unsterilized ☐

Q11) How many animals are there in the herd? Male_____ Female _____

Q12) How many animals in the herd are showing clinical signs of mastitis? _____

Q13) What clinical signs have you seen in the affected cows?

i) Reduction in milk yield ☐
ii) Clotted milk ☐
iii) Blood in milk ☐
iv) Reduction in feed intake ☐
v) Swollen udder ☐
vi) Others (specify) _____
_____

Q14) Has any treatment been given to the animals? Yes ☐ No ☐

Q15) If yes, was it prescribed by a Veterinarian? Yes ☐ No ☐

Q16) If no, where did you get the medicine from? _____
_____

Q17) Date of treatment: (*Or how many weeks ago*) _____

Q18) What drugs were used in treatment?

i) _____
ii) _____
iii) _____

Q19) What effect(s) has the treatment shown since?

i) No improvement ☐
ii) Slight improvement ☐
iii) Marked improvement ☐
iv) Worse than before ☐
v) Other (specify) _____
_____

Thank you for taking your time to fill this questionnaire.