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Original research

Development of a prototype milk screening reagent for the detection of bovine subclinical mastitis using common detergents and *Hibiscus sabdariffa* petals

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ABSTRACT

Mastitis is an inflammatory condition of the mammary gland of female animals which can either be clinical or subclinical. Subclinical mastitis is that which cannot be diagnosed by physical assessment of the udder, however, it continues to reduce the quality and quantity of the milk leading to economic losses. Diagnosis and treatment of subclinical mastitis is therefore of paramount importance to the farmer, however, the commercially available California Mastitis Test (CMT) reagent commonly used to detect subclinical mastitis is expensive and not readily accessible especially in the rural agrarian communities. This work was conducted to develop a test reagent from locally and readily available materials that can be used by rural farmers in the detection of subclinical mastitis. Different concentrations (1.5%, 3% and 6%) of different household detergents dissolved in water extract of *Hibiscus sabdariffa* (zobo) have been explored for their suitability to detect somatic cells in cow milk. The reagents were mixed with mastitic and nonmstatitic milk while the test conducted with CMT served as control to which the results from formulated reagents were compared for sensitivity. It was found that a 3% detergent concentration in *zobo* can be used to obtain gel formation and a colour change from red to dark green corresponding to somatic cell counts and pH respectively, much like the CMT reagent. The test conducted revealed 53.57%, 46.42%, 42.85% and 42.85% of cows' fore-quarter milk samples were positive to CMT, detergents A, B and C respectively. The combination of household detergent and water extract of *Zobo* can therefore be an alternative to CMT reagent in the detection of subclinical mastitis, especially in the rural areas. This indicates the potential of using these test reagents for subclinical mastitis screening. Further studies are suggested to standardize and patent this reagent as an alternative for subclinical mastitis diagnostic test.

KeyWords: Subclinical mastitis, household detergent, *Hibiscus sabdariffa.*

INTRODUCTION

Mastitis is a disease of economic importance in the livestock industry owing to its interference with milk production and high cost of treatment (Hogeveen *et al.*, 2019; Cheng & Han, 2020). Predisposing factors such as poor management, hygiene, teat injuries and faulty milking machines are known to play significant roles in the entry of the infectious agents and the course of the disease (Zenebe *et al.*, 2014).

In Nigeria, management and diseases particularly mastitis, constitute major constraints to effective dairy husbandry system (Shittu *et al.*, 2008; Adamu *et al.*, 2020). The economic impact of mastitis has been widely encountered by most livestock farmers; however, determining the actual cost is very challenging. The economic loss due to mastitis at herd level can be classified into four main sources attributable to loss due to depreciation in the milk quality, reduced milk production especially in chronic sub-clinical infection, loss due to discarded milk, cost of veterinary services, and treatment, as well as losses incurred due to increase replacement rate or culling of cows during their productive years (Adamu *et al.*, 2020).

The primary aim of herd health programme is to minimize economic loss due to disease. Thus, early diagnosis and treatment of disease conditions is paramount to a profitable animal husbandry. Mastitis is the most widespread infectious disease and from an economic presepctive, also the most important in dairy cattle (Sharma *et al.*, 2012; Tiwari *et al.*, 2000).

California Mastitis Test (CMT) is a simple cow-side indicator test for subclinical mastitis based on milk somatic cell count estimation. In this test, the reagent (a detergent) lyses the cell wall of all somatic cells in the milk thereby gelling the DNA in those cells (Middleton *et al.*, 2004; Whyte *et al.*, 2005)

The consequent increase in viscosity of the reagent when added to milk sample is directly related to the relative number of somatic cells. On the basis of the viscosity

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change, the sample can be semi-quantitatively scored to allow for sample comparison and to facilitate communication of the severity. The reaction is scored on a scale of 0 (where mixture remains unchanged) to 3 (solid gel formation) with a score of 2 or 3 being considered a positive result. Concurrent to evaluating the change in viscosity, the CMT reagent also contains a pH indicator that will turn from purple to yellow in acidic milk. Although the CMT kit is not very expensive and does not require skilled personnel to run as routine test for dairy animals, the availability of the CMT kit seems to be a challenge for livestock producers especially rural farmers. Moreover, the commercially available CMT reagent has potential of harming individuals when there is accidental contact with the eyes resulting to sharp pain, tearing, swelling and blurred vision as well as skin irritation with redness and pain following contact.

There are several other techniques routinely used in the diagnosis and screening of milk sample, but all these techniques are either too technical for a rural farmer to employ, too expensive for the farmer or not sensitive enough. Accessibility of testing reagent and ease of diagnosis are fundamental to patronage of a particular testing kit especially in rural agrarian communities of developing nations.

In view of the aforementioned, this study was designed to determine the potential application of common household detergents as substitute to the conventional CMT reagents during the detection of subclinical mastitis and also the use *Hibiscus sabdariffa* petals (*Zobo*) as an alternative to the CMT pH indicator.

MATERIALS AND METHODS

STUDY AREA

The study was conducted in Sokoto, Sokoto State. Sokoto State is located in the Northwestern part of Nigeria with a land area of approximately 28,232.37 square kilometers and located on latitude 13°N and between longitudes 4.8°E and 6.54°E. It is bordered to the North by Niger Republic, to the East by Zamfara State and to the South and West by Kebbi State. The State is blessed with livestock resources and is ranked second in livestock population in Nigeria (Iloeje, 2001).

EXTRACTION OF *HIBISCUS SABDARIFFA* (*ZOBO*) FOR USE AS PH INDICATOR

Petals of the *Hibiscus sabdariffa* plant were grinded into fine powder and one gram (1g) was weighed. It was then soaked in 100 ml of distilled water at room temperature for 24 to 48 hours and filtered using Whitman's filter paper size 1.0.

RECONSTITUTION OF 0.02% PHENOL RED INDICATOR

This was done according to the manufacturer's instruction (Ward's Science®, New York), by weighing 0.02g of Phenol Red powder and dissolving it in 100 ml of distilled water.

VISUAL ASSESSMENT OF PH DETERMINATION ABILITY OF PHENOL RED AND *HIBISCUS SABDARIFFA* FLOWER (*ZOBO*)

The ability of the pH indicators to change color under acidic or alkaline conditions was tested by a series of procedures. Three milliliters of 0.02% Phenol Red and 3 ml of 1% *Zobo* extract were measured in separate test tubes. The colors of Phenol Red and that of *Zobo* extract were visually identified and recorded. To test for color change under acidic condition, 1 ml of 0.5 Molar HCl was added into each of the test tubes containing the solutions until a colour change is visually observed. To test for color change under alkaline condition, 1 ml of 0.5 Molar NaOH was added into each of the test tubes containing the solutions until a colour change is visually observed. Reversal of the pH color indication was done by adding 0.5 Molar NaOH where 0.5 Molar HCl was initially used and vice versa.

PREPARATION OF TEST REAGENTS:

For each of the detergents used for this experiment, three concentrations (1.5%, 3% and 6%) were reconstituted by separately using Phenol Red and Zobo extract as diluents. The protocol for the reconstitution is further described. For 1.5% concentration, 1.5 g of detergent was weighed and dissolved in 100 ml of Phenol Red solution or dissolved in 100 ml of Zobo extract, as the case may be. For 3% concentration, 3 g of detergent was weighed and dissolved in 100 ml of Phenol Red solution or dissolved in 100 ml of Zobo extract, as the case may be. The same was done to get 6% concentration.

MILK SAMPLE COLLECTION

Milk samples were aseptically collected from four dairy farms located in the Sokoto metropolis. Milk samples were collected from each of the four quarters of the cow's udder after scrubbing with disinfectant directly into the sample container.

ASSESSMENT OF SAMPLES

Before commencement of the experiment, all milk samples collected were subjected to California mastitis test and White Side test in order to determine their actual status with respect to somatic cell count. This is in order to have a positive and negative control samples that will be used as guide to the test procedure.

TEST PROCEDURE

To determine the somatic cell counts and pH of the samples, equal volumes (3 ml each) of the test reagent and milk

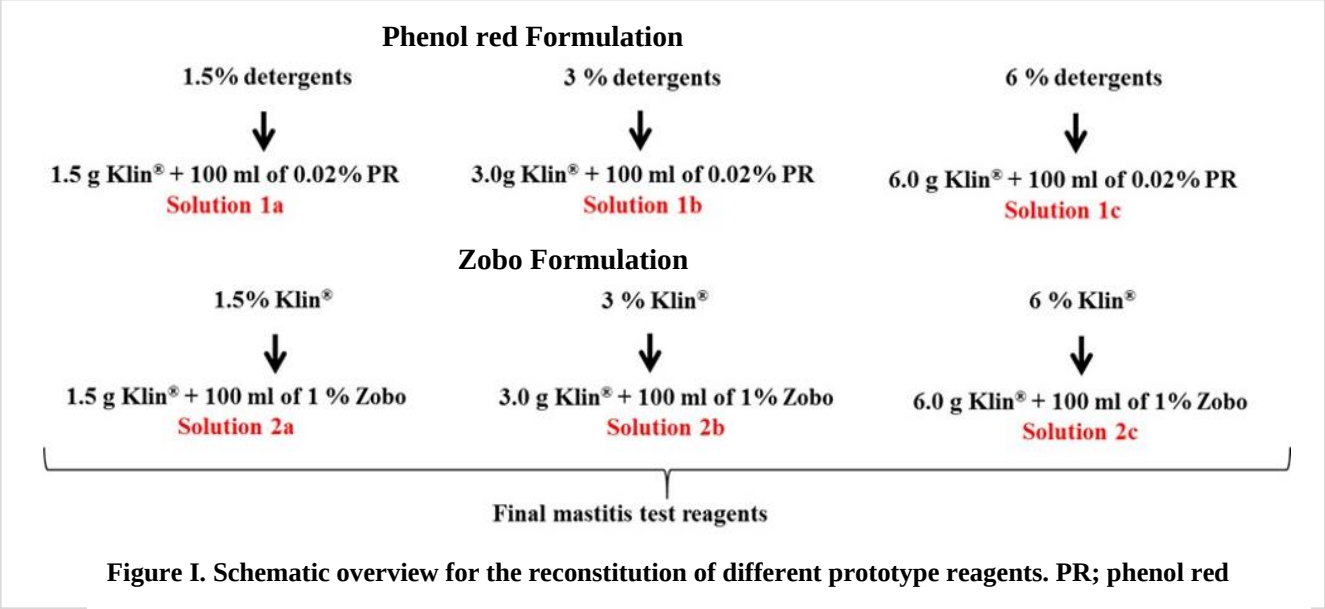

Figure I. Schematic overview for the reconstitution of different prototype reagents. PR; phenol red

sample were thoroughly mixed by gentle rocking in a CMT paddle. CMT reagent (BOVIVET CMT Liquid®, Denmark) and distilled water were used for positive and negative controls respectively.

The mixture was gently rocked for about 30 seconds during which gel formation and color change were observed.

After subjecting the milk samples to varying concentrations of detergents, assessments were conducted by five independent assessments groups of three individuals each.

EXPERIMENT 1 GROUPINS FOR TESTING MASTITIC MILK

In experiment I, there are 8 groups of experimental testing reagents (Table I). Group I contained the negative control in which a mastitic milk was mixed with 3 ml of distilled water. The positive control contained CMT as testing reagent for a mastitic milk. Others (groups 2-7) contained different components of testing reagents as highlighted in Figure I and 3 ml of mastitic milk.

EXPERIMENT II GROUPINGS FOR TESTING NON-MASTITIC MILK

In experiment II, there are 8 groups of experimental testing reagents (Table II). Group I contained the negative control in which a non-mastitic milk was mixed with 3 ml of dH_2O+pH indicator. The positive control contained 3 ml of CMT reagent and 3 ml non-mastitic milk. Others (groups 2-7) contained different components of testing reagents as highlighted in Figure I using 3 ml of non-mastitic milk.

RESULTS

pH INDICATORS

The pH of *Zobo* is acidic and visually assumes a red colour, when 1 ml of 0.5 Molar HCl was added this colour remained unchanged. However, when 1 ml of 0.5 Molar NaOH was added to the *Zobo* extract, it changed colour to blue or dark green as shown on Figure II.

The pH of phenol red immediately after reconstitution is alkaline and assumes red colour, and the addition of 1ml 0.5

Table I : Eight groups of experiment I and their component testing reagents

Groups	Composition of testing reagent
Group 1	Negative control => 3 ml of distilled water + 3 ml of known mastitic milk
Group 2	3 ml of Solution 1a + 3 ml of known Mastitic milk.
Group 3	3 ml of Solution 1b + 3 ml of known Mastitic milk.
Group 4	3 ml of Solution 1c + 3 ml of known Mastitic milk.
Group 5	3 ml of Solution 2a + 3 ml of known Mastitic milk.
Group 6	3 ml of Solution 2b + 3 ml of known Mastitic milk.
Group 7	3 ml of Solution 2c + 3 ml of known Mastitic milk.
Group 8	Positive control => 3 ml of CMT reagent + 3 ml of Mastitic milk.

Table II : Eight groups of experiment II and their testing reagent components

Groups	Components of testing reagents
Group 1	Negative control => 3 ml (dH_2O+pH indicator) +3 ml Non mastitic milk.
Group 2	3 ml of Solution 1a + 3 ml of Non mastitic milk.
Group 3	3 ml of Solution 1b + 3 ml of Non mastitic milk.
Group 4	3 ml of Solution 1c + 3 ml of Non-mastitic milk.
Group 5	3 ml of Solution 2a + 3 ml of Non-mastitic milk.
Group 6	3 ml of Solution 2b + 3 ml of Non mastitic milk.
Group 7	3 ml of Solution 2c + 3 ml of Non mastitic milk.
Group 8	Positive control => 3 ml of CMT reagent + 3 ml of Non mastitic milk.

Molar NaOH deepened the red colour. However, when 1ml of 0.5 Molar HCl was added to it, the colour changed from red to yellow as shown in Figure III.

All the reconstituted reagents were tested and results were

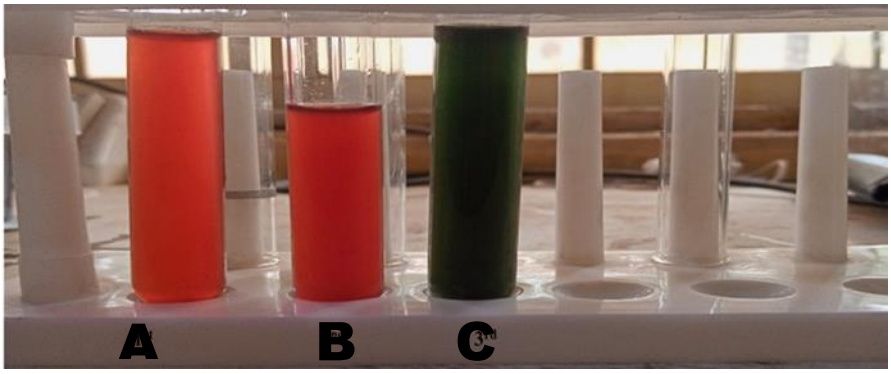

Figure II. *Hibiscus sabdariffa* "zobo" aqueous extract showing red colour in test tube A at acidic pH when 1ml of 0.5 Molar HCl was added, test tube B showing natural red colour when nothing was added, while test tube C shows colour change to dark green indicating alkaline pH after adding 1ml of 0.5M NaOH.

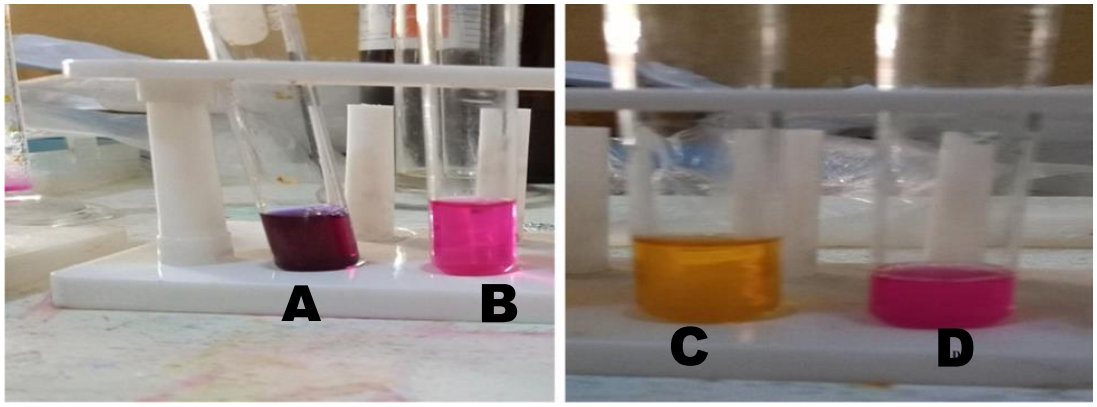

Figure III. Reconstituted phenol red in test A showing dark red colour upon addition of 1ml 0.5Molar NaOH indicating alkaline pH, in test tubes B and D indicating red colour immediately after reconstitution, in test tube C showing yellow colour after adding 1ml of 0.5Molar HCl indicating acidic pH.

Figure IV. Showing colour changes by adding the test reagent to fermented milk. A_1 and A_2 showing deep yellow colouration of acidic milk before and after gentle rocking respectively, upon addition of CMT reagent; B_1 and B_2 showing dark red colouration of acidic milk before and after gentle rocking respectively upon addition of *zobo* reagent, while C_1 and C_2 showed acidic milk before and after gentle rocking respectively upon addition of phenol red reagent

evaluated by the five different assessment groups subjectively.

Decisions were made based on simple majority verdict by the panel of assessors. At the end of the experiments, it was unanimously agreed that all the detergents used can react and form gel in the presence of somatic cells, however, brand A detergent was found to give better results that are most similar to that obtained using CMT kit. It was equally observed that 3% concentration of all the tested detergents yielded results most similar to that obtained using CMT kit, this is shown in Figure IV.

ASSESSMENT OF FRESH MILK SAMPLES USING THE FORMULATED REAGENTS.

Twenty-eight fresh milk samples collected from 14 lactating cows were subjected to detection of somatic cells with the formulated reagents, using CMT reagent as positive control. The test was conducted according to instructions for performing CMT test. The result is as shown on Table III.

Table III. Reactions of milk with CMT reagent and household detergents A, B, C (Klin®, Ariel® and Good Mama®) respectively.

S/NO.	SAMPLES	CMT REAGENT	A	B	C
1	A1	-	-	-	-
	A2	-	-	-	-
2	B1	++	+	+	+
	B2	++	++	++	++
	B3	+	+	+	+
3	C1	+	+	+	+
	C2	+++	+++	+++	++
	C3	+++	+++	++	+++
4	D1	-	-	-	-
	D2	-	-	-	-
5	E1	-	-	-	-
	E2	-	-	-	-
6	F1	+	+	+	+
	F2	+	+	-	+
7	G1	-	-	-	-
	G2	-	-	-	-
8	H	-	-	-	-
9	I	++	++	+	++
10	J	++	++	+	+
11	K1	+	-	-	-
	K2	-	-	-	-
12	L1	+	+	+	+
	L2	+	+	+	+
13	M1	-	-	-	-
	M2	-	-	-	-
	M3	-	-	-	-
14	N1	+	+	+	-
	N2	+	-	-	-

Key: A = Klin®, B = Ariel®, C = Good mama® . ¬¬- = Negative, + = Positive (mild), ++ = Positive (moderate), +++ = Positive (severe)

DISCUSSION

The need to detect mastitis at its subclinical form is essential to the success of mastitis control and treatment. Culture method and somatic cell count are the basic procedures for diagnosis of mastitis, but the reagents needed to screen cows before milking are not readily available to small scale farmers in rural areas.

In the present study, the test conducted revealed 53.57%, 46.42%, 42.85% and 42.85% of cows' fore-quarter milk samples were positive to CMT, detergent A, detergent B and detergent C respectively. This implies that the accuracy, sensitivity and specificity of test reagents used in this study were almost similar to that of the commercially available CMT mastitis test kit. Importantly, the costing and ease of access of the prototype reagents was lower as compared to commercially available mastitis test. Previous studies have demonstrated that screening of milk to detect subclinical mastitis is one of the most accurate methods (Kabir *et al.*, 2019). Therefore, the newly developed milk screening reagent can be an independent, cheap and farmer friendly and alternative subclinical mastitis screening testing reagent readily available for use by rural livestock owners.

The use of zobo as indicator of colour change in these reagents is attributable to anthocyanin pigments it contains. The same reason is responsible for the use of zobo as a stabilizing colour change in preparation of juice (Ukwubile *et al.*, 2013).

Furthermore, the research conducted showed that the household detergents can be used in the detection of subclinical mastitis. And out of the three different detergents used in this research, Detergent 'A' produced result that is most similar and consistent to that conducted with CMT reagent. This research agrees with the findings of Muhammad *et al.* (1995) and Karabasanavar *et al.* (2021), who used surf excel, a detergent, as a cheaper, user-friendly alternative cow side subclinical mastitis diagnostic test.

The use of detergent and zobo to detect mastitis in dairy milk is a new initiative. Hence, there is dearth of literature on this theme. Different detergents have been used for disinfecting milking instrument prior to milking in dairy cattle with possible contamination of milk with the detergents. The study of Romero *et al.,* (2014) showed that the presence of acid and alkaline detergents in goat's milk did not produce a great interference in the microbial tests, except in high concentrations of detergents that could cause non-compliant results, while these concentrations are difficult to find in practice with standard cleaning procedure.

CONCLUSION

The diagnostic efficiency of household detergents is very similar to CMT in detection of subclinical bovine mastitis, Therefore, it is reasonable to suggest that detergents A, B and C at optimized concentrations can be used as alternative cow-side subclinical mastitis diagnosis test. This indicates the potential of using these test reagents for mastitis screening. Further studies are suggested to standardize and patent these reagents as an alternative for subclinical mastitis diagnostic testing.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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