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EVALUATION OF THREE (3) TECHNIQUES IN THE DIAGNOSIS OF SUBCLINICAL MASTITIS WITH ANTIBIOTIC RESISTANCE OF *S. AUREUS* IN NIGERIA

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ABSTRACT

This study was aimed at evaluation of two diagnostic techniques and the cultural isolation of *Staphylococcus aureus* for the testing of subclinical mastitis in lactating cows. A total of 144 milk samples from 36 cows were examined. Ten (10) ml milk samples were aseptically collected from each quarter into labelled sterile universal bottles. The California Mastitis Test (CMT) and the Methylene Blue Reduction Test (MBRT) were carried out on each sample, before cultural isolation (gold standard test) was conducted. Forty eight milk samples were CMT-positive and 60 samples were MBRT-positive, while the gold standard (*S. aureus* isolation) had 31 positives. The 89.5 % samples of CMT-positive were correctly identified by the test culture. The proportions of samples that tested negative for Subclinical Mastitis (SCM) that did not have the disease, which is a negative predictive value, are 97.9 % and 92.9 % for CMT and MBRT, respectively. The highest total resistance of *S. aureus* to antibiotics was detected for gentamycin and chloramphenicol (100 %), followed by streptomycin and amoxicillin at 80.6 % and 74.1 %, respectively. The control and monitoring of subclinical mastitis in lactating cows are of great importance to human health. A good management system constitutes the priority in controlling subclinical mastitis in lactating cows.

Key words: bacterial culture; CMT; MBRT; subclinical mastitis

INTRODUCTION

Mastitis is a common disease of cattle worldwide. It can result in significant welfare issues for cattle and also results in huge economic losses through milk wastage, treatment costs and loss of productivity [24]. Early and accurate identification of the organisms involved in mastitis can offer great benefits in controlling the disease at the farm level.

Mastitis can be categorised based on major causative pathogens which are contagious or environmental [14]. Contagious mastitis is caused by the spread of bacteria from the infected udder to a healthy cow. Cow-to-cow transmission of pathogenic bacteria takes place during milking [3]. Pastoralist's hands or milking machines can be major reservoirs of contagious bacteria. *Staphylococcus aureus, Streptococcus agalactiae* and *S. dysgalactiae* are examples of contagious pathogens [23]. In contrast, environmental mastitis originates from environmental elements such as soil, manure, water and bedding. Environmental mastitis is essentially a hygienic matter; which means pathogens from these environments can be eliminated by increasing the hygienic conditions of farms. Gram-negative bacteria

such as *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes*, and *Pseudomonas aeruginosa* are very important aetiologies of environmental mastitis [22].

Mastitis has also been classified based on clinical signs and symptoms in cows which are clinical or subclinical mastitis [22]. Clinical mastitis (CM) is the abnormality observed on the udder such as show redness, swelling, high temperature or pain as reported by Mpatswenumugabo et al. [17]. CM could be mild or severe. In mild clinical mastitis, the milk produced is characteristic of clots, colour changes, or consistency while in severe cases, fever, anorexia and/or shock may be observed [2]. Subclinical mastitis (SCM) is an infection without obvious clinical signs. SCM has an elevated somatic cell count that is more than 200,000 cells.ml^{-1} cut-off point [5]. This type of mastitis can be more dangerous because it can persist for the entire lactation or life of the animal [1, 26]. In addition, SCM could be ignored by farmers because it is asymptomatic; this may lead to economic losses due to low milk yield and quality and even culling of animals [23].

Detection of subclinical mastitis can be done using different techniques such as the California Mastitis Test (CMT), Methylene Blue Reduction Test (MBRT), Somatic Cell Count (SCC), bacterial culture and others [12].

The CMT is a simple cow-side indicator of the somatic cell count of milk. It works by rupturing the cell membrane of any cells in the milk sample, enabling the DNA in those cells to interact with the test reagent and produce a gel. It offers a practical method for identifying subclinical mastitis [12].

The MBRT is based on the fact that the colour imparted to milk by adding a dye such as methylene blue will disappear more or less quickly [8]. The removal of the oxygen from milk and the formation of reducing substances during bacterial metabolism causes the colour to disappear. The oxygen utilization is conducted by bacteria. Although certain bacterial species have far more of an impact than others, it is widely accepted that the more bacteria there are in milk, the faster the oxygen will be used, which will cause the colour to change more quickly [8]. As a result, the period of reduction is used to estimate the number of organisms in milk, although it is more likely to represent the complete metabolic processes taking place on the bacterial cell surface.

Mastitis is usually diagnosed by bacterial culture of milk samples as a standard technique. However, the logistic and financial considerations involved with sampling all fresh cows have precluded this technique from being widely adopted. The bacteriological culture of milk identifies the presence of mastitis pathogens but does not provide a measure of the degree of inflammation associated with the infection.

This study was aimed at evaluation of two diagnostic techniques known as the California Mastitis Test (CMT), the Methylene Blue Reduction Test (MBRT) and the cultural isolation of *Staphylococcus aureus* in testing subclinical mastitis in lactating cows.

MATERIALS AND METHODS

Study location

This research was carried out at a livestock investigation department with samples collected from Vom and Bukuru communities in Plateau State, Nigeria. The research area is Jos South Local Government area of Plateau State. It is located between latitude 08° 24N and Longitude 008°32', the postal code of the area is 930. Temperature ranges from 21–25 °C (70–77 °F), and from mid-November to late January, night temperature drops as low as 11 °C (52 °F). There is usually about 1,400 millimetres (55 inches) of rainfall annually in Jos, the precipitation arising from both conventional and geographic sources.

Study Population

Three (3) herds of cows chosen for this study had 23, 17 and 12 dairy cows individually. Two breeds of dairy cows were sampled: White Fulani and cross-bred cows. White Fulani cows are medium-sized animals with long horns. Their long and lyre-shaped horns effectively characterize them. They weigh around 250–380 kg whereas the cross-breeds weigh 450 kg on average. A White Fulani dairy cow produces around 6 litres of milk per day whereas cross-breed bovines can produce 30 litres per day. Fifty-two well-structured questionnaires were used to collect livestock information such as breed, age, parity, lactation stage, housing conditions – floor types, and husbandry system.

Physical examination and preparation of the udder

In order to exclude cows with clinical mastitis, a veterinarian clinically examined individual cows after being properly restrained. The shape, size, abnormal changes in milk, consistency, and temperature of the udder, were findings that were considered a clinical mastitis case. Subclinical mastitis was detected only based on the CMT and MBRT tests. The udders of cows without clinical mastitis including the teats were washed with clean water and dried. Then the teats were vigorously swabbed with cotton wool soaked in 70 % ethanol before milk sample collection to remove dust particles of bedding from the surface of the teats [18].

Sample size determination

The formula to calculate the sample size of cross-sectional studies:

$$N = \frac{z^2(pq)}{d^2}$$

Where:

N = minimum sample size of coliform isolates of milk samples

Z = standard error at 95 % Confidence Interval (1.96)

p = local prevalence of 18.1 % [2] = 0.181

q = 1.0 - p = 0.819

d = degree of accuracy (5 %) = 0.05

$N = ((1.96)^2 \text{ X } 0.181 \text{ X } 0.819)/(0.05)^2$

N = (3.8416 X 0.1482)/0.0025 = 227.78

Sample collection

A total of 144 milk samples from 36 cows were examined. Ten (10) ml milk samples were aseptically collected from each quarter into labelled sterile universal bottles [25]. The samples were kept at 4 °C in a cooler and transported to the microbiology laboratory of the Federal College of Animal Health and Production Technology, Vom, Nigeria.

Diagnostic tests

I) California Mastitis Test (CMT)

The CMT was conducted according to Dingwell et al. [9]. It was conducted by adding equal amounts of CMT reagent and milk from each quarter on the test paddle and rotating for 10 seconds. Samples with a CMT score of 0 or T (trace) were considered negative while those with CMT scores of 1+ (mild clumping), 2++ (moderate clumping), or 3+++ (heavy clumping) were considered positive for subclinical mastitis.

II) Methylene blue reduction test (MBRT).

Two (2) ml of milk was transferred into a test tube and labelled appropriately, then 1ml of redox indicator, methylene blue was added to each test tube containing the milk sample. The test tube mouth was tightened with stoppers, then the tube was gently inverted about 4–5 times to ensure proper mixing of the methylene blue solution. The test tubes were kept in the water bath at 37 °C. The incubation time was noted. That is, the time elapsed for the colour to turn whitish appearance and finally, the test tubes were stabilised for 5 minutes.

$$\text{Sensitivity} = \frac{TP}{TP+FN} \text{ x100}$$

$$\text{Specificity} = \frac{TN}{TN+FP} \text{ x100}$$

$$\text{Positive predictive value} = \frac{TP}{TP+FP} \text{ x100}$$

$$\text{Negative predictive value} = \frac{TN}{TN+FN} \text{ x100}$$

$$\text{Accuracy} = \frac{TP+TN}{TP+FP+FN+TN} \text{ x100}$$

Where: TP = True Positive; FP = False Positive; TN = True Negative; FN = False Negative.

The following diagnostic test characteristics were determined using the milk bacteriological culture result as a gold standard control.

III) Microbial cultural examination

One (1) ml of each milk sample was inoculated into 9 ml nutrient broth for enrichment and incubated at 37 °C overnight. After that, *S. aureus* colonies that showed as yellow colonies on a red medium (MSA) were identified by streaking a loopfull of the broth culture on that medium. Many bacteria could be isolated from SCM milk samples such as *S. aureus, Streptococcus agalactiae, Streptococcus dysgalactiae Escherichia coli, Klebsiella pneumonia, Klebsiella oxytoca, Klebsiella aerogenes* [21] but to compare SCM tests, *S. aureus* was chosen because it is one of the most prevalent bacteria isolated from SCM [22].

Antibiotics susceptibility testing

According to the recommendations of the Clinical and Laboratory Standards Institute guidelines [7], the Muller-Hinton Agar (MHA), and the (Oxoid, Basingstoke, Hampshire, England), the plate technique was used in a

standardized Kirby-Bauer disk diffusion method. The McFarland standards of the test organism were inoculated on the surface of the already prepared agar plate. The plates were allowed to stand for 30 minutes to allow effective diffusion and then incubated at 37 °C for 18–24 hours. Then, zones of growth inhibition were measured and noted to the closest millimeter. The following antibiotic discs were used: ciprofloxacin (5 μg); norfloxacin (10 μg); gentamycin (30 μg); amoxycillin (10 μg); streptomycin (30 μg); rifampicin (5 μg); erythromycin (15 μg); chloramphenicol (30 μg); ampiclox (30 μg); levofloxacin (5 μg). The plates were incubated at 37 °C for 24 hours in Mueller–Hinton agar (MHA). An inhibition zone diameter of each antimicrobial was then measured and interpreted as sensitive (S), intermediate (I), and resistant (R) by comparing with the *mecA* negative (*S. aureus* ATCC 29213) and *mecA* positive (ATCC 33591) controls included in each test run. Inhibition zones were interpreted according to the CLSI guidelines [7].

Data analysis

The percentage accuracy of the tests and sensitivity, specificity, and the predictive values of the CMT, and MBRT compared to bacterial growth was calculated using the standard two-by-two contingency tables. The data were also analysed by Chi-square test (χ^2) to observe the significant association between the subclinical mastitis test, and the gold standard. $P < 0.05$ was considered statistically significant.

RESULTS

From the 144 milk samples tested for SCM in lactating cows, 48 milk samples were CMT positive and 60 samples were MBRT positive, while the gold standard (bacterial culture) had 31 positives (Table 1). There was a significant difference ($P < 0.05$) between the results of bacterial culture (gold standard) and the two tests, CMT and MBRT. Tables 2 and 3 are two-by-two tables comparing the gold test results and those of the two other SCM tests. Both tables showed significant differences ($P < 0.05$). Table 4 demonstrated that despite 46 and 60 samples being SCM positive with CMT and MBRT, the test's accuracies were 78.5 % and 71.5 %, respectively. There was no significant difference between the accuracy of the positive MBRT and its corresponding CMT result ($P > 0.05$).

In Table 5, the sensitivity of CMT, which is the proportion of the samples that are correctly identified by the test culture, is 89.5 %. The specificity, which is the proportion of samples that do not have SCM, and were correctly identified as negative, is 76.8 %. The positive predictive value, which is the proportion of samples which tested positive for SCM, and have the disease was 36.9 %. The negative predictive value which is the proportion of samples that tested negative for SCM, and do not have the disease was 97.9 %. The sensitivity and specificity of MBRT reached 80.6 % and 69.0 %, respectively. The positive predictive value and negative predictive value for MBRT were 41.7 % and 92.9 %, respectively. There was no significant difference between the results of CMT/bacterial cultural test and the MBRT/bacterial cultural test ($P > 0.05$).

Table 6 shows the total antibiotic susceptibility of *S. aureus* isolates, with 100 % resistance to gentamycin and chloramphenicol followed by streptomycin (80.6 %) and amoxicillin (74.1%). The *S. aureus* isolates showed the highest sensitivity to ampiclox (42.0 %), followed by rifampicin (29.0 %), and levofloxacin (29 %). There was a statistically significant difference ($P < 0.05$) between the antibiotics used and their sensitivity or resistance efficacy.

Table 7 shows the risk factors associated with subclinical mastitis in the lactating cows sampled. It includes breed, age, parity, lactation stage, floor type. and the husbandry system used. Of the ninety-two quarters of the examined cross-bred cows, 40 were found positive for SCM, while of 52 examined White Fulani cows' quarters only 8 were positive. The SCM positivity in 6–7 years old cows was higher than in the younger animals. Higher positivity (38) was detected in cows in mid lactation stage compared to those in early lactation stage (10). Cows bred in muddy farms had more SCM positive animals (35), than those bred on farms with concrete floor (13). On farms with semi-intensive husbandry system the SCM positivity was higher compared to the extensive system of rearing. No significant differences between the variables were detected for individual risk factors.

Table 1. Prevalence of subclinical mastitis tests

Tests	No of samples	No positive (%)	χ^2	P-value
Culture	144	31 (21.5)	13.51	0.001
CMT	144	48 (33.3)		
MBRT	144	60 (41.7)		

**P < 0.05

Table 2. Two-by-two table of CMT and culture (gold standard) results

CMT	Culture		Total	χ^2	P-value
	Positive	Negative			
Positive	29	17	46	68.87	0.000***
Negative	2	96	98		
Total	31	113	144		

***P < 0.05

Table 3. Two-by-two table of MBRT and culture (gold standard) results

MBRT	Culture		Total	χ^2	P-value
	Positive	Negative			
Positive	25	35	60	24.65	0.000***
Negative	6	78	84		
Total	31	113	144		

***P < 0.05

Table 4. Percentage accuracy of various indirect tests used for the diagnosis of SCM

Test	Samples examined	Positive samples (%)	TP (%)	FP (%)	TN (%)	FN (%)	Accuracy (%)	χ^2	P-value
CMT	144	46 (31.9)	17 (11.8)	29 (20.1)	96 (66.7)	2 (1.4)	78.5	10.35	0.114
MBRT	144	60 (41.7)	25 (17.4)	35 (24.3)	78 (54.2)	6 (4.2)	71.5		

TP = True Positive; FP = False Positive; TN = True Negative; FN = False Negative

Table 5. Comparing the screening parameters of CMT/culture and MBRT/culture

Test	CMT/Culture (%)	MBRT/Culture (%)	χ^2	P-Value
Sensitivity	89.5	80.6	1.39	0.845
Specificity	76.8	69.0		
Positive predictive value	36.9	41.7		
Negative predictive value	97.9	92.9		
Accuracy	86.8	71.5		

P > 0.05

Table 6. Antibiotic susceptibility of *Staphylococcus aureus* isolates

Antibiotic used	Sensitivity (%)	Intermediate Sensitivity (%)	Resistant (%)	χ^2	P-value
Ciprofloxacin	0 (0.0)	25 (80.6)	6 (19.4)	214.39	0.000***
Norfloxacin	7 (22.6)	12 (38.7)	12 (38.7)		
Gentamycin	0 (0.00)	0 (0.00)	31 (100.0)		
Amoxicillin	2 (6.5)	6 (19.4)	23 (74.1)		
Streptomycin	0 (0.0)	6 (19.4)	25 (80.6)		
Rifampicin	9 (29.0)	18 (58.1)	4 (12.9)		
Chloramphenicol	0 (0.0)	0 (0.0)	31 (100.0)		
Ampiclox	13 (42.0)	5 (16.0)	13 (42.0)		
Levofloxacin	9 (29.0)	9 (29.0)	13 (42.0)		

***P < 0.05

Table 7. Risk factors associated with SCM in lactating cows

Risk factor	Category	No. of cows' quarters examined	SCM positive	Prevalence (%)	χ^2	P-Value
Breed	Cross	92	40	15.4	11.7	0.000
	White Fulani	52	8	43.5		
Age	5–6	52	3	15.4	27.8	0.000
	6–7	92	45	43.5		
Parity	1–2	144	48	33.3		
Lactation stage	Early	28	10	35.7	0.1	0.766
	Mid	116	38	32.8		
Floor-type	Concrete	52	13	25.0	2.5	0.111
	Muddy	92	35	38.0		
Husbandry system	Semi-intensive	52	27	51.9	12.6	0.000
	Extensive	92	21	22.8		

***P < 0.05

DISCUSSION

Mastitis in dairy farms is common with substantial economic losses, including poor fertility, the increased abortion rate in early pregnancy, premature culling rates, and decreased milk yield and composition [6]. It could pose a threat to human health since it may be responsible for zoonoses and food toxin infections [10]. Effective control and eradication programs for mastitis depend on a good management system, identification of carrier cows, and accurate medication [13].

Of the 144 quarters of cows sampled, the gold standard (bacterial culture) had the lowest SCM prevalence of 21.5 % compared to CMT and MBRT which had 33.3 % and 41.7 %, respectively. There was a statistical significance between the three. B a s t a n et al. [4] and S a i d i et al. [21] showed a similar trend with a lower prevalence for bacterial culture compared to the CMT, although the prevalences were higher than those of this study. Both CMT and culture were positive for 29 samples, unlike MBRT and culture which had 25 positive samples. B a s t a n et al. [4] showed a higher 212 positive samples for CMT and culture out of 344 samples. The positive bacterial growth did not present as CMT or MBRT positive possibly due to the low sensitivity of the two test methods or to elimination by the animal's defences, such as phagocytosis, which could produce a negative result [16]. L a n g o n i et al. [16] observed a higher frequency of the bacterial agent *Staphylococcus* spp. than the other bacterial agents, corresponding to 35.53 %.

The sensitivity of the CMT in this study is similar to that of D i n g w e l l et al. [9] who recorded a sensitivity of 82.4 % but with a much higher specificity (80.6 %) than that of this study which was 1.5 %. While a CMT with low specificity may be a concern to a researcher, it can be thought of as being too keen to find a positive result, even when it is not present, and may give a high number of false positives. This could result in a test indicating that a cow without SCM has the disease, even when it is not present.

The sensitivity of MBRT, in this study, was higher than that of CMT. G u n a w a n et al [11] were in agreement with a sensitivity of 77 %. G u n a w a n reported a lower specificity, higher positive predictive value, and lower negative predictive value of 50 %, 94 %, and 18 % which are higher than the ones recorded in this study (69.0 %, 41.7 %, and 92.9 %, respectively).

Amoxicillin (which is in the class of penicillins) and streptomycin are frequently used for the treatment of mastitis and had already been used in previous treatments, which led to their high resistance on the *Staphylococcus aureus* samples, which might be related to the resistance induced by previous administrations. R i b e i r o et al. [20] in a study on pathogenic microorganisms in bovine milk, found the highest resistance rates of 53.5 % when the strains were submitted to penicillin. Similarly, R e n et al. [19] investigated the prevalence and antimicrobial susceptibility of antimicrobial susceptibility of *Staphylococcus aureus* from subclinical bovine mastitis in dairy farms located in China. The authors found the resistance rates to penicillin, and tetracycline, in dairy farms located in China. The authors found that the resistance rates to penicillin, tetracycline, and chloramphenicol were 58.5 %, 18.5 %, and 1.5 %, respectively.

Between the two breed groups, there was a statistically significant prevalence of mastitis. This might be as a result of White Fulani breeds' superior climatic adaptability as opposed to crossbreeds brought into the country from temperate regions. The results of this study corroborate those of other authors' publications, which claim that native breeds are less vulnerable to bovine mastitis than exotic breeds like crossbreeds [15]. There was statistical significance for age and husbandry system as risk factors of subclinical mastitis.

CONCLUSIONS

The CMT was the most accurate test after bacterial culture, followed by MBRT. It is important to take into consideration that laboratory tests such as bacterial culture and MBRT require adequate laboratory facilities, personnel and time. CMT is a reliable diagnostic method for use as a regular screening test in field conditions even by less trained dairymen. The control and monitoring of subclinical mastitis in lactating cows are of great importance to human health. A good management system constitutes the priority in controlling subclinical mastitis in lactating cows. *Staphylococcus aureus* is one of the most frequently identified bacteria in milk samples. The use of penicillin (10 µg), tetracycline (30 µg), or amoxicillin (10 µg) was not effective in inhibiting mastitis-causing pathogens *in vitro*.

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