Antibiotic Susceptibility of Mastitogenic Bacteria Isolated From Clinical Mastitis Cows in Midlands Province, Zimbabwe

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SUMMARY

Mastitis is a global challenge for the dairy industry and mastitogenic bacteria play a critical role among other causes. Many mastitogenic bacteria are becoming resistant to single or combination antibiotic therapy, making mastitis cases difficult to cure. Nevertheless, there is insufficient evidence on the occurrence and antibiotic resistance patterns of mastitogenic bacteria in commercial dairy and communal farms in the Midland Province of Zimbabwe that might support a holistic approach to mastitis mitigation. A cross-sectional study aimed to isolate and evaluate the antibiotic susceptibility patterns of mastitogenic bacteria from cattle with clinical mastitis was conducted. A total of 164 milk samples were analyzed, of which 36.6% (60) samples were collected from commercial farmers, and 63.4% (104) came from communal farmers. The samples were cultured on standard media and sensitivity patterns of the identified bacteria were tested against 14 antibiotics using the Kirby-Bauer disc diffusion method. All milk samples from communal farms were positive for mastitogenic bacteria compared to 88% (53) of mastitogenic positive milk samples from commercial farms. The most common microorganisms from all the 157-mastitogenic positive milk samples were *Staphylococcus aureus* (37.5%) and *Escherichia coli* (23.3%). The highest resistance was observed against Penicillin, Erythromycin, Ampicillin, and Lincomycin, whereas most isolates were susceptible to Sulphamethoxazole, gentamycin, neomycin, kanamycin, cloxacillin, ertapenem, ceftriaxone, Amp-Ampicillin, amikacin, vancomycin, and tetracycline. Futher research to investigate the significance of resistant mastogenic bacteria in terms of Veterinary costs, production losses and potential public health transmission of antibiotic resistant mastogenic bacteria is recommended.
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Keywords: *Cows, Mastitogenic bacteria, Antibiotic susceptibility testing, mastitis milk*

INTRODUCTION

Mastitis (clinical, subclinical, and pathological changes that include inflammation of the mammary glands) has resulted in the reduction of total milk production, the quality of milk (high somatic cell and microorganism counts), and substantial financial loss to dairy farmers (Sharma et al., 2010; Yang et al., 2011). Mastitis is caused by mastitogenic microorganisms, including bacteria, mycoplasmas, yeasts, and algae (Vásquez-garcía et al., 2017; Zadoks et al., 2011).

More than 135 mastitogenic bacteria have been identified and classified into host-adapted or contagious and environmental pathogens (Gitau et al., 2014). The two main bacterial species classified as host-adapted microorganisms are *Streptococcus agalactiae* and *Staphylococcus aureus.*

Mastitogenic organisms are sometimes found in the cow's environment, including coliforms and Streptococci (Shaheen et al., 2016; Sharma, 2010). Bacteria that cause environmental mastitis were isolated from the udder, drinking water, and milking machine wash water, and these include Enterobacteriaceae such as *Klebsiella* species, *Escherichia coli, Citrobacter* species, *Proteus* species, and *Salmonella* (Katsande et al., 2013; Silva and Costa, 2001).

Staphylococcus aureus causes most clinical and subclinical mastitis, reducing milk production by nearly 50%, and has gained resistance to antibiotics making it persistent (Jones et al., 2009). On the other hand, *Streptococcus agalactiae* is one of the major causes of contagious mastitis coupled with milk production cessation.

Dairy cows infected with *Streptococcus agalactiae* show no clinical signs and can only be identified when high bulk tank somatic cell and bacteria counts are noticed (Shaheen et al., 2016). During the latent phase, *Streptococcus agalactiae* can spread insidiously throughout the herd (Gitau et al., 2014).

Another cause of contagious mastitis is *Corynebacterium bovis*, which causes mild udder infections, a mild increase in somatic cell count, and a slight reduction in milk production (Harjanti et al., 2018).

Streptococcus agalactiae and *Corynebacterium bovis* rarely cause clinical mastitis. Non-aureus Staphylococcus (NAS) species are also important causative agents of bovine mastitis worldwide.

Staphylococcus haemolyticus and *Staphylococcus chromogenes* are the prominent NAS associated with bovine mastitis cases (Hosseinzadeh et al., 2014). Although NAS has been isolated from both clinical and subclinical mastitis cases (Kudinha et al., 2002), it is commonly associated with subclinical mastitis (Katsande et al., 2013 Harjanti et al., 2018). Environmental bovine mastitis has been attributed to the members of the family Enterobacteriaceae, and these can cause the sharing of resistance through the mobile genetic elements.

Antibiotics have been used for several years without eradicating mastitogenic bacteria, and there is an increase in antibiotic resistance against pathogens, causing mastitis (Zdolec et al., 2016). The prolonged incorrect use and overuse of antibiotics in the absence of antibiotic susceptibility screening have led to treatment failures, high treatment costs, and antimicrobial resistance (Anholt et al., 2017). In line with this, the concerns of dairy farmers, veterinarians, public health, and researchers are anchored on poor treatment outcomes, the presence of multidrug-resistant mastitogenic bacteria, and the consumption of raw unpasteurized milk and milk products containing multidrug-resistant bacteria (Shaheen et al., 201).

Treatment of mastitis should consider mastitogenic bacteria's ability to exhibit varying degrees of antibiotic susceptibility patterns. Accordingly, a critical step in curbing mastitis is identifying mastitogenic

bacteria (Shaheen et al., 2016) and analyzing their antibiograms (Gitau et al., 2014).

However, currently, there is limited information on the prevalence and antibiotic resistance pattern of mastitogenic bacteria in commercial dairy and communal farms in the Midlands province, Zimbabwe.

The study aimed to isolate mastitogenic bacteria associated with bovine mastitis' clinical symptoms in the Midlands Province and evaluate their antimicrobial susceptibility patterns.

MATERIALS AND METHODS

Study area

Midlands Province (Supporting Figure 1) covers 49,166 km^2 and is divided into 8 districts: Chirumhanzu, Gokwe North and South, Gweru, Kwekwe, Shurugwi, Zvishavane, and Mberengwa. Dairy farming in Midlands Province is divided into commercial and communal production systems, and this province lies in agro-ecological regions III and IV of Zimbabwe.

Communal and commercial farmers in the province encounter many constraints, including a high prevalence of diseases and parasites, a low management level and limited forage availability, and poor marketing management. Although the most common diseases reported by farmers are blackleg, heart-water, babesiosis, anthrax, and anaplasmosis, mastitis still contributes to heavy loss in dairy farming. Generally, in Zimbabwe, including Midlands province, there are challenges such as unavailability and high cost of drugs and inadequate veterinary officials (Tavirimirwa *et al.*, 2013).

Study design and sampling

This was a cross-sectional study and was approved by the National Animal Research Ethics Committee Medicines Control Authority of Zimbabwe. Milk sampling was performed between December 2019 and June 2020 on 50 (62.5%) communal (dairy smallholder) and 30 (37.5%) commercial farmsdepending on accessibility, cows with clinical mastitis symptoms, and sample collection by the farmers. A convenience sampling method was used to collect 164 milk samples from 164 cows showing clinical mastitis symptoms.

Of the 164 milk samples, 36.6% (60) samples were collected from commercial farmers, and 63.4% (104) came from communal farmers. Cows showing clinical mastitis such as redness, swelling, pain, heat, and hardness of the teats were sampled from each of the 8 districts (Supporting Table 1).

All milk teats were disinfected using 70% alcohol, and the first three streams of milk were discarded to minimize contamination with bacteria from the skin around the teat canal (Katsande et al., 2013). Milk samples were then collected aseptically using sterile containers and immediately transported on ice packs to the Central Veterinary Laboratories for microbiological isolation and identification of the mastitogenic bacteria.

Milk samples were collected from each quarter (milk teat) showing clinical signs of mastitis into an independent, labelled and sterile container, and notably, the majority of cows had one teat infected, some two, some three and very few cows had all the teats infected. Having seen this discrepancy, an equal amount of milk sample from those cows with more than one teat infected was then aliquot and mixed aseptically to make a composite milk sample before cultivation and considered a single sample.

Isolation and identification of bacteria in milk

For identifying present mastitogenic bacteria, 10 µl of each milk sample was streaked on 5 % Sheep-Blood Agar (Oxoid)

and MacConkey Agar (Oxoid) using the quadrant streaking method (Harjanti et al., 2018). After 24 hours of incubation at 37 ± 2ºC, microbiological deductions were implemented on the growing colonies. The agar plates were examined by colony morphology and hemolysis pattern. A sample showing 2 to 3 colonies of similar morphology were considered positive for mastitogenic bacteria and was analyzed further. More than one colony type was described as mixed growth, but more than three colony types were regarded as contamination. Individual colonies from samples with two or three different colony types were subcultured at 37 ± 2ºC for 18-24 hours to obtain pure cultures for gram staining and biochemical testing.

Sterilized milk was used as a negative control. For biochemical tests, *Staphylococcus aureus* ATCC 33862, *Streptococcus agalactiae* ATCC 12386, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Citrobacter freundii* ATCC 8090, and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains.

Colony morphology (on sheep-blood agar and McConkey agar), gram staining, and biochemical tests (Supporting Figure 2) were used to identify the bacteria to genus and or species name with the aid of the reference books available at the laboratory.

Antimicrobial sensitivity testing

The Kirby-Bauer agar disc diffusion method was performed without any replicates following Clinical and Laboratory Standards Institute (CLSI; Hombach et al., 2013). The selection of antimicrobial discs was based on the antibiotics available and those commonly used in veterinary practices in Zimbabwe.

A suspension of each bacterial isolate (of approximately $1–2 \times 10^8$ CFU/mL) was made to compare with a 0.5 McFarland organism in standard saline solution and plated on a Mueller Hinton agar using a sterile swab. Antibiotic impregnated discs (Oxoid) were applied onto the plates using an antibiotic multichannel disc dispenser.

Antibiotics used were Ertapenem 10µg, Vancomycin 30µg, Amikacin 30µg, Ampicillin 10µg, Gentamycin 30µg, Penicillin G 10 µg, Erythromycin 15 µg, Tetracycline 30µg, Ceftriaxone 30µg, Kanamycin 30µg, Neomycin 10µg, Cloxacillin 5µg, Lincomycin 15µg and Sulfamethoxazole 25µg (Oxoid, Germany). After incubation (24 h at 37 ± 2° C), zones of growth inhibition around each of the antibiotic discs were measured to the nearest millimeter. Results were interpreted as resistant, intermediate, and sensitive to the different antibiotics used, using the Clinical and Laboratory Standards Institute (CLSI) breakpoints (Girma et al., 2012; Hombach et al., 2013).

The number of samples showing resistance was expressed as a percentage.

Data Analysis

Overall, bacterial prevalence rates were calculated as the number of times the bacteria were detected divided by the total number of times all the bacterial isolates were detected and multiplied by 100.

Results were also stratified according to the type of farms and prevalence rates calculated as the number of milk with bacterial growth divided by the total number of milk from that farm multiplied by 100.

Bacterial growth from the two farms was used to compare any significant difference in bacterial growth between the two farms using the X^2 Test at a 95% confidence interval using R-Software.

RESULTS

Bacterial isolates

Out of the 164 milk samples collected from commercial and communal farms, 95.7% (157 of 164) were positive for bacterial growth. From commercial farms, 88% (53 out of 60 milk samples) were positive for bacterial growth, and all milk samples collected from communal farms (100%) were positive for bacterial growth (104 out of 104).

Table 1. Prevalence of the isolated mastitogenic bacteria

Bacteria identified	Frequency (F)	OP (%)	CI	P(F)	CI	P(C)	CI
Escherichia coli	80	23.3	15.7-33.0	14.5 (50)	8.5-23.3	8.7 (30)	4.2-16.5
Staphylococcus aureus	129	37.5	28.2-47.8	30.5 (105)	21.9-40.6	7 (24)	3.1-14.4
Corynebacteria spp	21	6.1	2.5-13.2	5.2 (18)	2.0-12.1	0.9 (3)	0.03-6.1
Klebsiella pneumoniea	29	8.4	4.0-16.1	5.8 (20)	2.3-12.9	2.6 (9)	0.6-8.6
Citrobacter freundi	15	4.4	1.5-11.0	3.8 (13)	1.2-10.2	0.6 (2)	0.02-5.6
Streptococcus agalactiae	3	0.9	0.03-6.1	0.9 (3)	0,03-6.1	0 (0)	0
Staphylococcus intermedius	12	3.5	1.0-9.8	3.2 (11)	0.9-9.4	0.3 (1)	0-5.1
Proteus mirabilis	20	5.8	2.3-12.9	5.5 (19)	2.2-12.5	0.3 (1)	0-5.1
Pseudomonas aeruginosa	10	2.9	0.7-9.0	2 (7)	0.3-7.7	0.9 (3)	0.03-6.1
Enterobacter aerogenes	10	2.9	0.7-9.0	2.3 (8)	0.5-8.2	0.6 (2)	0.02-5.6
Non-aureus *Staphylococci*	15	4.4	1.5-11.0	2 (7)	0.3-7.7	2.3 (8)	0.5-8.6
No Growth after 24/48hours	7	4.3	1.4-10.9	0		4.3 (7)	
Samples with bacterial growth	157	95.7	89.1-98.5	63.4 (104)		32.3 (53)	
Commercial farms with growth	53/60	88	79.6-93.4				
Communal farms	104/104	100	95.4-100				

95% Confidence intervals (**CI**), low-high, Frequency: number of times the bacteria was detected, OP: Overall prevalence (%) of detection, P(F): Prevalence rates (%) at communal farms and (F: number of times the bacteria was detected in communal farms), P(C): Prevalence rates at commercial farms and (C: number of times the bacteria was detected in commercial farms)

There was a statistically significant difference between milk samples' proportion with mastitogenic bacteria from commercial and communal farms (X^2 = 11.834, p-value = 0.0005817).

The most probable isolates found were *Staphylococcus aureus (S. aureus), Streptococcus agalactiae (S. agalactiae), Staphylococcus intermedius (S. intermedius), and Klebsiella pneumoniae (K. pneumoniae*), *Enterobacter aerogenes (E. aerogenes), Citrobacter freundi (C. freundi), Pseudomonas aeruginosa (P. aeruginosa), Non-aureus Staphylococci (NAS), Proteus mirabilis (P. mirabilis), Escherichia coli (E. coli) and Corynebacterium bovis (C. bovis).* Table 1 showed the isolated mastitogenic bacteria, and their prevalence *Staphylococcus aureus* (37.5%) was the most prevalent bacteria, and *Streptococcus agalactiae* (0.9%) was the least prevalent mastitogenic bacteria (Table 1).

Table 2. Resistance percentages for the isolated bacteria against tested antibiotics

Isolate	N	Antibiotics													
		Pen	Sxt	Ery	Gen	Neo	Kan	Clo	Erta	Ceft	Amp	Ami	Van	Tet	Lin
Citrobacter	15	47	0	100	0	100	100	0	100	0	100	0	0	100	100
Corynebacterium	21	62	0	100	0	100	100	100	100	0	100	100	0	62	100
E. coli	80	100	0	65	10	100	0	65	65	65	96	1	100	80	100
Enterobacter	10	100	30	100	0	100	0	60	100	100	20	100	50	100	100
Klebsiella	29	100	0	7	0	100	7	7	7	7	100	100	100	69	100
Proteus	20	0	0	0	0	0	0	0	100	100	0	0	0	100	100
Pseudomonas	10	40	20	40	0	60	30	50	100	100	60	0	52	40	100
Streptococcus	3	33	0	100	0	0	33	0	0	0	100	0	0	0	100
S. aureus	129	67	40	48	0	0	47	16	0	69	47	0	0	86	100
S. intermedius	12	0	0	100	0	0	0	0	0	0	100	0	0	0	100
NAS	15	0	0	100	0	0	0	0	0	0	100	0	0	0	100
Overall resistance	344	67	17	57	2	47	30	31	38	53	70	18	35	75	100

Key: Pen: Penicillin, Sxt: Sulphamethoxazole, Ery:Erythromycin, Gen:Gentamycin, Neo:Neomycin, Kan:Kanamycin, Clo:Cloxacillin, Erta:Ertapenem, Ceft:Ceftriaxone, Amp:Ampicillin, Ami:Amikacin, Van:Vancomycin, Tet:Tetracycline, and Lin:Lincomycin.

Antimicrobial susceptibility testing

The isolated mastitogenic bacteria showed variable antibiotic resistance rates ranging from 0 to 100% (Table 2). Gentamicin was the most effective antibiotic against the mastitogenic bacteria isolates, with all mastitogenic bacteria susceptible except for only 10% resistant *Escherichia coli* isolates.

Most mastitogenic bacteria were resistant to penicillin with microorganisms; *E. coli, K. pneumoniae*, and *E. aerogenes* recording 100% resistance.

Though penicillin was least effective, the percentage of penicillin-resistant microorganisms was less than 100% for *S. aureus* (67%), *C. bovis* (62%), *C. freundi*

(47%), *S. agalactiae* (33%), and *P. aeruginosa* (40%).

Mastitogennic bacteria are relatively susceptible to Sulphamethoxazole as indicated by 0% of the isolated *S. aureus*, 30% of *E. aerogenes* and 20% of *P. aeruginosa*, which are resistant against Sulphamethoxazole, while the rest of the isolated bacteria were susceptible to Sulphamethoxazole. *Proteus mirabilis* was the only bacterium showing 100% susceptibility to Erythromycin, while the rest of the mastitogenic bacteria had varying percentages of resistance. All the bacteria isolates were susceptible to gentamycin except 10% resistant *Escherichia coli* isolates. All the coliforms and *C. bovis* (100%) and *P. aeruginosa (*60%) showed resistance to neomycin. *Pseudomonas aeruginosa* (30%), *Streptococcus agalactiae* (33%), and *Staphylococcus aureus* (47%) were resistant to kanamycin**.**

Varying percentages of resistance were also observed against cloxacillin, ertapenem, ceftriaxone, ampicillin, amikacin, and tetracycline (Table 2). Notably, all the isolated mastitogenic bacterial isolates were resistant to lincomycin.

DISCUSSION

The mastitogenic bacteria identified in this study corroborates with the findings of studies conducted in and outside Zimbabwe (Adane et al., 2012; Akhoon, 2012; Demme et al., 2015; Garedew et al., 2012; Gweshe et al., 2020; Haq et al., 2009; Junaidu et al., 2011; Katsande et al., 2013; Kudinha et al., 2012; Lakshmi et al., 2016; Mbuk et al., 2016; Rahman et al., 2013; Tufani et al., 2012; Zahid, 2004).

Staphylococcus aureus was the most prevalent mastitogenic bacteria in this study (Table 1), and this is in agreement with findings obtained in other studies (Getahun et al., 2008; Sori et al., 2005).

The high prevalence of *S. aureus* can reflect the level of management, hygiene practices, and implementation of standard biosecurity measures in the milking parlour, like washing hands between milking cows and teat dipping (Belachew, 2016).

Poor animal husbandry practices are usually responsible for establishing and spreading *S. aureus* infection. Contaminated hands, cloth, milking equipment, and house flies can help spread *S. aureus* infection within the herd. Non-aureus Staphylococci (NAS) and *C. bovis* are historically considered to be of little importance and are often described as minor pathogens (Kudinha et al., 2002).

The impact of NAS infection is increasing, probably because significant pathogens decrease (Sampimon *et al*., 2009). The NAS has been shown to provide a protective influence on the udder against superinfection by *E. coli, Streptococcus agalactiae*, or *S. aureus* (Kudinha et al., 2002). However, this is not in agreement with the present study results as there was a simultaneous presence of NAS, *E. coli*, and *S. aureus* in some of the milk samples. Mastitogenic bacteria causing environmental mastitis were more abundant than those causing contagious mastitis indicating poor environmental/farm management practices and poor hygiene practices.

The high prevalence of mastitogenic bacteria in this study can be attributed to the milk sampling strategy, which targeted only cows with clinical symptoms of mastitis.

Usually, the prevalence rates of different mastitogenic bacteria are attributed to: a) the level of management being implemented at farm level (Communal farms, 100%, had higher prevalence rates than commercial farms, 88% but is inferior in management level).

There was a statistically significant difference in terms of the presence of mastitogenic bacteria in mastitis milk from commercial and communal farms (X-squared = 11.834, p-value = 0.0005817); b)

early identification and treatment of clinical cases; c) culling of carriers, geographical variances, milking techniques, the season of study, adaption of a mastitis control program and d) herd sizes and hygienic levels maintained in different dairy herds (Adane et al., 2012; Markey et al., 2013).

Growth was absent in 7 (4.3%) milk samples from cows with clinical cases of mastitis, and this negative results on culture can be attributed to: a) the elimination of the etiological agent in chronic cases or from reduced presence in milk and b) mastitis of viral origin, un-cultivatable bacterial species or mycoplasma, tick bites or treatment in progress (Kateete et al., 2013; Makovec et al., 2003).

Surveillance information concerning progressive changes in the susceptibility patterns of mastitogenic bacteria to antibiotics used to treat mastitis is essential (Mekonnen et al., 2005). It is prudent to base the decision of antimicrobial therapy for mastitis on veterinary consultation(s) supported by laboratory antimicrobial susceptibility test results. This reduces the danger posed by prolonged uninformed use of antimicrobials as it increases the selection of resistant bacteria and the acquisition of drug resistance (Breser et al., 2018).

Antimicrobial susceptibility patterns may differ depending on bacterial species tested and geographical region (Markey et al., 2013). Other bacteria, such as Staphylococci, acquire resistance easily (Markey et al., 2013). The resistance mechanisms differ per each organism isolated and antibiotic used, and these mechanisms include mutations, efflux pumps, chromosomal and plasmid-borne integrons (Breser et al., 2018).

For example, penicillin resistance is usually due to the production of beta-lactamase enzymes by the gram-negative bacteria, which leads to the distortion of the antibiotic structure rendering the drug ineffective.

Erythromycin resistance results from mutations at the antibiotic binding site, and high levels of resistance to this antimicrobial are also due to its wide usage. High resistance rates of mastitogenic bacteria to penicillin were also observed in studies carried out by Kateete and colleagues (Kateete et al., 2013).

The resistance of *E. coli* to penicillin is regularly high worldwide (Markey et al., 2013) and has been worsened due to the frequent intramammary infusions of the drug (Kateete et al., 2013). According to Maggs (2008), ampicillin is often effective against *E. coli* and *Proteus* spp, but in the present study, this was true for *P. mirabilis* and not valid for *E. coli* (showed a high resistance rate to ampicillin of 96%).

The resistance of *P. aeruginosa* to penicillin, ampicillin, amoxicillin-clavulanic acid, tetracycline, macrolides, trimethoprim-sulfamethoxazole, and cephalosporin was also observed in other studies (Breser et al., 2018; Markey et al., 2013). Low levels of resistance were observed for the aminoglycosides (gentamycin (2%), amikacin (18%), neomycin (30%) and kanamycin (47%)), sulphonamides (Sulphamethoxazole (17%), glycopeptides (vancomycin 35%)) and carbapenems (ertapenem (38%).

Of particular concern is the development and dissemination of carbapenem-resistance bacteria in livestock associated with grave implications for treatment options in human medicine. This has necessitated continued monitoring of carbapenem susceptibility among bacteria from food-producing animals.

However, it is essential to note that the selection of antibiotics for treatment based on in-vitro susceptibility testing does not guarantee success in-vivo (Schwarz et al., 2010).

In conclusion, the study revealed that *Enterobacter aerogenes, C. freundi, K. pneumonia, P. aeruginosa, P. mirabilis, E. coli, S. aureus*, NAS, and *C. bovis* were the bacterial strains associated with bovine mastitis in the Midlands province, and they

showed some resistance against the commonly used veterinary antibiotics.

Most of the isolated bacterial species were environmental pathogens, highlighting poor environmental management in the region.

The emergence of antibiotic-resistant bacteria is a public health concern, and one health approach in managing and reducing antibiotic resistance should be enforced regularly. In veterinary medicine, the focus should be on infection prevention through good animal husbandry, acceptable hygiene/sanitation practices, and vaccines such as the autogenous vaccine against *S. aureus* mastitis in cows, which reduces the severity of clinical and subclinical mastitis (Markey et al., 2013).

Although the study revealed antimicrobial resistance, there is a need to conduct further research with a larger sample size of cows. In addition to a larger sample, useful information such as animal breeds, antibiotics source if used, and farmers' education and mastitis control training should be included in future studies. It is of paramount importance for further research to focus on more sensitive molecular antimicrobial resistance and phylogenetic analysis of antibiotic-resistant mastitogenic bacteria to understand the antibiotic susceptibility of mastitogenic bacteria.

Moreover, preventive measures against mastitis can involve vaccine production and administration, reducing antibiotics overuse, and preventing antibiotic resistance in mastitogenic bacteria.

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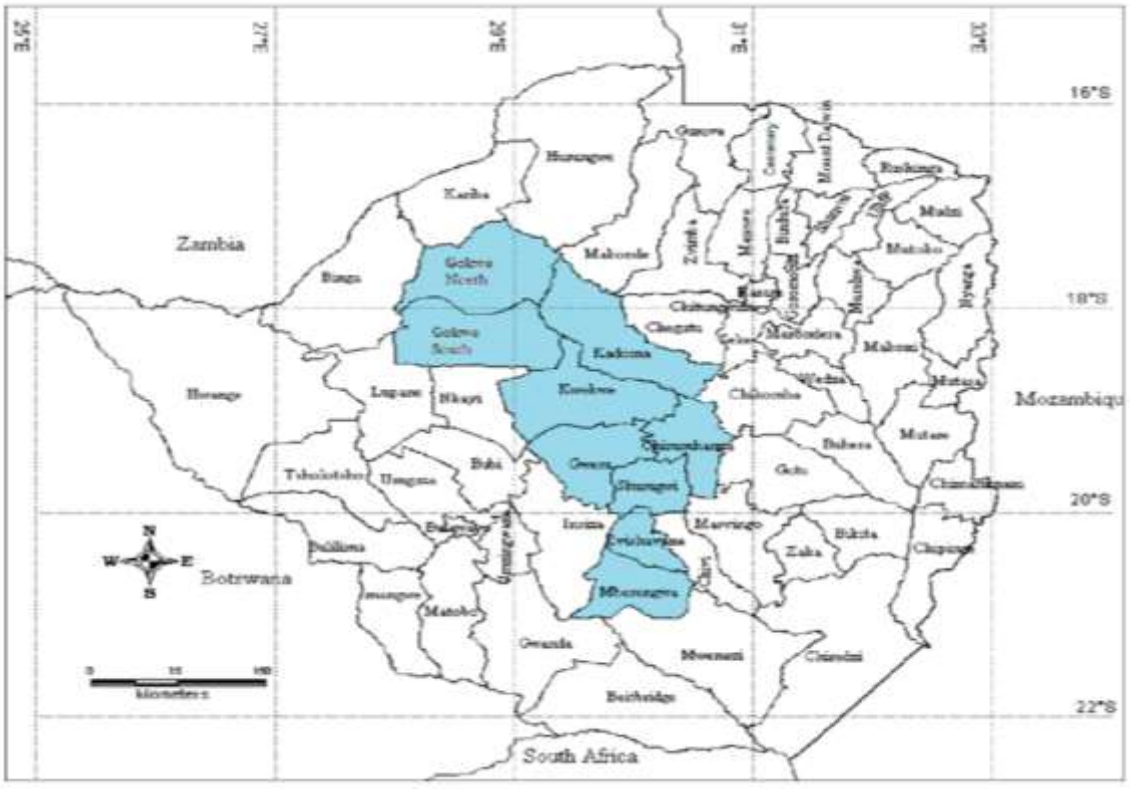
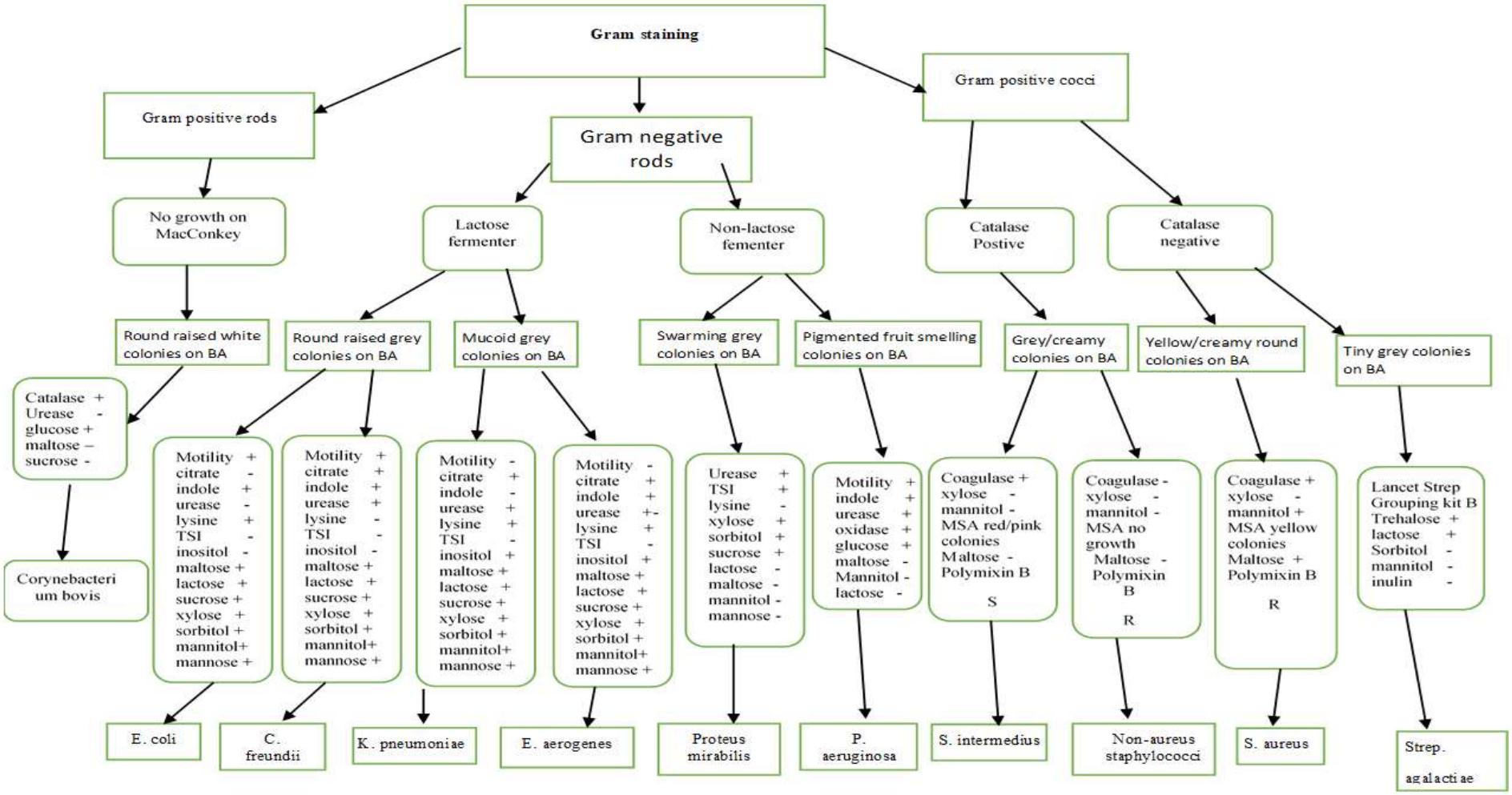
SUPPLEMENTARY INFORMATION

Classification of bacteria

Bacteria were classified according to gram-staining as gram-negative rods, gram-positive rods, and gram-positive cocci. All gram-negative rods were further classified based on colony morphology on McConkey agar into lactose and non-lactose fermenters. Those classified as lactose fermenters (LF) showed pink-red colonies, while non-lactose fermenters (NLF) were pale colonies. The LF was subjected to indole, motility, triple iron sugar, lysine decarboxylase, citrate, and carbohydrates (inositol, maltose, mannitol, lactose, xylose, sorbitol, sucrose, and mannose) fermentation tests, and the bacteria were identified as indicated in Fig 2. Colony morphology on sheep-blood agar was used to identify the LF bacteria, and mucoid grey raised colonies were regarded as *Klebsiella* and *Enterobacter* species (*Klebsiella* species are indole negative), raised round grey colonies were attributed to *Citrobacter* and *E. coli* (*E.coli* cannot ferment citrate). Those bacteria classified as gram-negative NFL were classified into two based on their morphology on sheep blood agar, with *Proteus mirabilis* being swarming grey colonies and *Pseudomonas auroginosa* showed pigmented colonies with a fruity smell. The small to medium-sized grey/yellow/creamy colonies on sheep blood agar and yielding Gram-positive cocci were subjected to catalase tests. All colonies that tested negative for catalase were identified as *Streptococci* and were subjected to Streptococcus grouping kit and were found to be in group B and further tested for sugars such as trehalose and identified as *Streptococcus agalactiae* (Fig 2). Catalase positive colonies were further tested for oxidase, and oxidase negative colonies were regarded as *Staphylococcus* species. The *Staphylococcus* species were further differentiated using coagulase test, sugar fermentation, polymyxin B (Fig 2) and were confirmed to be *S. aureus*, *S. intermedius*, and non-aureus staphylococci (NAS).



Suppoting Figure: Map showing the Midlands Province coloured in blue where samples were collected.



Supporting Figure 2: Flow diagram showing Gram staining and some biochemical tests used to identify different bacterial isolates in this study.

Supporting Table 1. Commercial and communal sample size distribution for each District

District	Number of samples and percentages : n (%)		
	Commercial farms	Communal farms	Commercial and communal farms
Chirumhanzu	8 (13.33)	10 (9.62)	18(10.98)
Gokwe North	10 (16.67)	20 (19.23)	30 (18.29)
Gokwe South	7 (11.67)	17 (`16.35)	24 (14.63)
Gweru	12 (20)	10 (9.62)	22 (13.41)
Kwekwe	7 (11.67)	13 (12.5)	20 (12.20)
Shurugwi	8(13.33)	10 (9.62)	18 (10.98)
Zvishavane	4 (6.67)	13(12,5)	17 (10.37)
Mberengwa	4 (6.67)	11(10.58)	15 (9.15)
Total	**60 (100)**	**104 (100)**	**164 (100)**