BMC Research Notes

RESEARCH NOTE Open Access

Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from bovine mastitis in North West Cameroon: public health implications

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Abstract

Objectives Methicillin-resistant *Staphylococcus aureus* (MRSA) is a zoonotic pathogen that poses a serious threat to veterinary and public health worldwide. We investigated mastitis milk samples for contamination with MRSA and also characterized the MRSA isolates by investigating antimicrobial resistance and virulence factors.

Result We confirmed MRSA in 69 of 201 (34.3%) *S. aureus* isolates recovered from a total of 300 samples. Of the 69 MRSA, 19 (27.5%) were from subclinical cases, while 50 (72.5%) were from clinical cases. The MRSA showed high resistance to penicillin (100%), ampicillin (100%), trimethoprim (69.6%), and tetracycline (69.6%) while susceptibility was observed for gentamicin (100%), vancomycin (95.7%), and ciprofloxacin (91.3%). Most isolates (65.2%, 45/69) were multidrug resistant. Thirteen antibiotypes (A_1-A_{13}) were identified and the most prevalent was A_8 ($TMP^RE^RTET^RAMP^RP^R$). All MRSA produced haemolysins, caseinase, and coagulase. Lipase, gelatinase and lecithinase were found in 97.1%, 94.2% and 91.3% of isolates respectively. Genotyping revealed *coa* (100%) and *spa* (68.1%) genes. We recommend educating dairy farmers on the public health implications of consuming unpasteurized raw milk and the implementation of proper hygiene practices in dairy farms.

Keywords Methicillin-resistant *Staphylococcus aureus*, Bovine mastitis, Antimicrobial resistance, Public health, Virulence factors, Cameroon

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Introduction

The contamination of products of animal origin, such as milk, by *Staphylococcus aureus* is a public health hazard [1]. This opportunistic zoonotic bacterium commonly colonizes the skin and mucosa of livestock, particularly dairy cows with subclinical or clinical mastitis. The bacterium causes various diseases in humans ranging from mild skin infections to systemic infections such as pneumonia and meningitis [2]. A strain of *S. aureus* that develops resistance to the antibiotic methicillin, is referred to as methicillin-resistant *S. aureus* (MRSA). Methicillin-resistant *Staphylococcus aureus* is a notorious virulent biovar considered a global public health threat [3].

Some studies have linked the high prevalence of MRSA contamination in dairy farms to excessive and empirical administration of antibiotics in the treatment of dairy cows and poor sanitation management during milking [4]. Contamination of milk can occur during collection from the udder and also from the hands of farmers during milking. Hence poor hygiene practices in dairy farms increase the risk of milk contamination with MRSA [4, 5].

Initially, MRSA was thought to be confined to the hospital environment where a mortality rate of up to 20% has been reported [6]. The burden of MRSA infections has additionally been amplified by the emergence and spread of community- and livestock-acquired MRSA leading to difficulty in defining the boundary between hospital–community-livestock transmission [7]. Strains of MRSA are a significant public health concern that affects humans and animals, with environmental contamination facilitating their spread. Addressing this problem is urgent and requires knowledge of its reservoirs in the healthy population, animals and the environment at national and international levels to support effective 'One Health' prevention and control strategies [8].

Several studies have reported the zoonotic transmission of this pathogen from pigs, poultry, cattle, and other livestock to farm workers, and other exposed people [9–11]. A very recent study demonstrated the zoonotic transmission of MRSA from cattle to humans via environmental interfaces through the detection of MRSA in cattle, human and environmental samples in the same farm vicinity in Bangladesh [12]. In Cameroon, very few studies have investigated MRSA in cases of bovine mastitis [13] and the livestock environment [3]. This study aimed to examine the level of MRSA contamination in dairy cow's mastitic milk and investigate the antimicrobial susceptibility of MRSA and the presence of some virulence factors, in order to understand the public health implications of consuming unpasteurized raw milk in the study area.

Materials and methods

Study area and data collection

The study was carried out in North West Cameroon, one of the most important cattle and milk production areas in the country [14]. The farms (with herd sizes ranging from 47 to 93), located at least 5 km from one another, were selected based on accessibility and farmers' willingness to participate. The selected farms represented the typical local dairy production practices for commercial purposes in the study area. Quarter milk samples were collected only from lactating cows with mastitis in at least one teat (12–24 in each farm) that had not received antibiotics within the past 15 days, in order to increase the chances of recovering *S. aureus*. Ancillary data (including age, breed, herd size, husbandry system, and consumption of raw milk) were also recorded for each cow. A trained veterinarian examined each cow for signs of clinical mastitis while subclinical mastitis was confirmed using the California mastitis test (ImmuCell1 CMT, Portland, USA).

We calculated the sample size using Thrusfield's formula of $N = \frac{Z^2 \times P(1-P)}{d^2}$ [15],

Where N = minimum sample size required, Z = 1.96 at a 95% confidence interval, P = expected prevalence of 11.1% of *S. aureus* contamination of milk and meat samples from a previous study in Cameroon [13] and d = desired absolute precision of 5%. This gave a sample size of 150, which was doubled in this study to increase precision [16].

Milk sample collection

Using aseptic techniques, the veterinarian collected ≈ 10mL quarter milk from the teat into a labelled screw-capped sterile plastic tube. The samples were transported on ice (4–8 °C) and stored at -20 °C in the Laboratory for Emerging Infectious Diseases, University of Buea, until needed for analysis.

Isolation and confirmation of *Staphylococcus aureus*

For isolation of *S. aureus*, 20µL of each sample were aseptically streaked on 5% sheep blood agar (Oxoid, Hampshire, England) and plates incubated aerobically at 37 °C for 24-48 h. Presumptive *S. aureus* colonies were purified on nutrient agar (Oxoid, Hampshire, England) followed by Gram staining, testing for catalase and coagulase production and phenotypic confirmation with API ID 20 STAPH gallery (bio-Merieux, France). For molecular confirmation of *S. aureus*, the *nuc* gene was amplified by PCR using primers previously described (Table 1). Unless otherwise stated, each PCR amplification was carried out in a 25µL final volume containing 5µL of DNA sample, 12.5µL of PCR mastermix (BioMix Red), 0.5µL of each primer (0.2µM), and nuclease-free water. A negative control (nuclease-free water replaced the DNA template) and

Table 1 Primers used in the study, sequences and expected amplicon sizes

Gene	Primer	Sequence (5'-3')	Size (bp)	Reference
nuc	nuc 1	AGTATATAGTGCAACTTCAACTAAA	450	[17]
	nuc 2	ATCAGCGTTGTCTTCGCTCCAAATA		
coa	coa F	ATAGAGATGCTGGTACAGG	600	[18]
	coa R	GCTTCCGATTGTTCGATGC		
mecA	mecA 1	GGCTATCGTGTCACAATCG	310	[19]
	mecA 2	CTGGAACTTGTTGAGCAGAG		
spa	spa F	AGACGATCCTTCGGTGAGC	200–600	[19]
	spa R	GCTTTTGCAATGTCATTTACTG		
etb	etb F	ACAAGCAAAAGAATACAGCG	226	[20]
	etb R	GTTTTTGGCTGCTTCTCTTG		
tst	tst F	ACCCCTGTTCCCTTATCATC	326	[20]
	tst R	TTTTCAGTATTTGTAACGCC		

a positive control (a previously identified *S. aureus* isolate stored in the laboratory [3] were included in each PCR run carried out in MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR cycling conditions for the *nuc* gene were 94 °C/5min, 40x [94 °C/1min, 58 °C/1min 72 °C/1min] and 72 °C/5min. Amplicons were electrophoresed on 1.5% high-resolution agarose gel stained with SYBR Safe DNA gel stain (Invitrogen). The amplified bands were visualized under ultraviolet light and photographed using Gel Documentation-XR (Bio-Rad, Hercules, CA, USA). Confirmed isolates were stored in nutrient broth supplemented with 20% glycerol at -70ºC for downstream assays.

Phenotypic identification and genotypic confirmation of MRSA

Using cefoxitin (30 µg) discs, the *S. aureus* isolates were screened to identify MRSA following the Clinical and Laboratory Standards Institute (CLSI) guidelines [21], MRSA strains were selected from the *S. aureus* isolates. The amplification of the *mecA* gene (see primers in Table 1) was confirmatory for MRSA and the PCR cycling conditions were the same as described above.

Antimicrobial susceptibility testing of MRSA

The Kirby-Bauer disc diffusion method was used to determine susceptibility and resistance to the following antimicrobials (Oxoid, England): vancomycin (VA, 30 µg), tetracycline (TET, 30 µg), penicillin (P, 10IU), ampicillin (AMP, 10 µg), streptomycin (STR, 10 µg), gentamicin (CN, 10 µg), erythromycin (E, 15 µg), trimethoprim (TMP, 5 µg) and ciprofloxacin (CIP, 5 µg). Each inoculum, adjusted to 0.5 McFarland standard, was seeded on Mueller-Hinton agar (HiMedia Laboratories, India); the antibiotics discs were placed on the plate and incubated at 35 °C for 16-18 h. For each isolate that showed resistance to vancomycin, the minimum inhibitory concentration (MIC) was determined using the agar dilution method. The antimicrobial susceptibility assays were repeated thrice as a quality control measure. Results were interpreted according to breakpoints provided by CLSI [21]. Antimicrobials were selected to represent different antimicrobial classes and also comprised those commonly used to treat staphylococcal infections in human and veterinary medicine.

Screening MRSA for some virulence factors

The ability of these isolates to produce hydrolytic enzymes was determined by inoculating TSA-1 medium (Bio-Rad, USA) supplemented with: 1% skim milk for caseinase, 1% gelatin for gelatinase, Tween 80 for lipase and 5% egg yolk for lecithinase. The presence of the hydrolytic enzyme was confirmed by a clear halo around the colonies. Growth on 5% sheep blood agar was used to detect haemolytic activity [22, 23].

Four genes; *coa* [18], *spa* [19], tst and *etb* [20] were investigated in the MRSA strains in uniplex PCR reactions. Primers used for the amplification of these genes are listed in Table 1. The *coa* and *spa* genes were amplified under the same conditions as the nuc gene described above except for the annealing step set at 51 °C/1min and 50 °C/1min for the *spa* and *coa* genes respectively. Amplification conditions for the *etb* gene were 94 °C/5min, 40x [94 °C/40s, 55 °C/40s, 72 °C/40s] and 72 °C/7min. The PCR conditions for the *tst* gene were: 94 °C/5min, 40x [94 °C/2min, 54 °C/2min, 72 °C/2min] and 72 °C/5min.

Ethical considerations

North West Regional Delegation of Livestock, Fisheries and Animal Industries approved the use of animals in the study. Oral consent was obtained from the dairy farmers.

Statistical analysis

Epi info version 2000 (Centers for Disease Control and Prevention, Atlanta, Ga.) was used for statistical analysis. Chi-square (χ^2) was applied to test whether associations between the distribution of MRSA and the possession of virulence genes were significant at p-value ≤ 0.05.

Table 2 The number of milk samples contaminated with MRSA from cows with clinical and subclinical mastitis

Mastitis status	Total milk samples analysed	Number positive for MRSA (%)	Pearson chi-square	*p*-values (95% CI)
Subclinical	93	19 (20.4)	0.503	0.478
Clinical	207	50 (24.2)		
TOTAL	300	69 (23.0)		

Table 3 Antimicrobials and their susceptibility testing results for MRSA identified in this study

Antimicrobial class	Antimicrobial agent	Number of isolates (*N* = 69)		
		Resistant (%)	Intermediate (%)	Susceptible (%)
Antifolate	Trimethoprim	48 (69.6)	00 (00)	21 (30.4)
Macrolide	Erythromycin	31 (44.9)	00 (00)	38 (55.1)
Tetracycline	Tetracycline	48 (69.6)	00 (00)	21 (30.4)
Penicillin	Ampicillin	69 (100)	00 (00)	00 (00)
	Penicillin G	69 (100)	00 (00)	00 (00)
Aminoglycoside	Streptomycin	9 (13.0)	7 (10.1)	53 (76.8)
	Gentamicin	00 (00)	00 (00)	69 (100)
Fluoroquinolone	Ciprofloxacin	6 (8.7)	00 (00)	63 (91.3)
Glycopeptide	Vancomycin	3 (4.3)	00 (00)	66 (95.7)

Table 4 Antibiotypes of MRSA identified in this study

Profile	Antibiotype	Multidrug resistant	Number of MRSA (%)
A_1	AMP^RP^R	No	15 (21.7)
A_2	$E^RAMP^RP^R$	No	1 (1.4)
A_3	$TET^RAMP^RP^R$	No	5 (7.3)
A_4	$TMP^RAMP^RP^R$	No	3 (4.4)
A_5	$TMP^RE^RAMP^RP^R$	Yes	1 (1.4)
A_6	$TMP^RTET^RAMP^RP^R$	Yes	11 (16.0)
A_7	$TMP^RAMP^RP^RSTR^R$	Yes	1 (1.4)
A_8	$TMP^RE^RTET^RAMP^RP^R$	Yes	23 (33.3)
A_9	$TMP^RTET^RAMP^RP^RSTR^R$	Yes	1 (1.4)
A_{10}	$TMP^RE^RTET^RAMP^RP^RSTR^R$	Yes	2 (2.9)
A_{11}	$TMP^RE^RTET^RAMP^RP^RCIP^RVA^R$	Yes	2 (2.9)
A_{12}	$TMP^RE^RTET^RAMP^RP^RSTR^RCIP^R$	Yes	3 (4.4)
A_{13}	$TMP^RTET^RAMP^RP^RSTR^RCIP^RVA^R$	Yes	1 (1.4)
TOTAL			69 (100)

Abbreviations E, erythromycin; TMP, trimethoprim; TET, tetracycline; CIP, ciprofloxacin; P, penicillin; STR, streptomycin; AMP, ampicillin; VA, vancomycin; ^{R}, resistance

Results

Overall, 300 samples were collected from 112 dairy cows in six farms. The same husbandry system, semi-intensive, was used in rearing these cows. The consumption of raw unpasteurized milk was reported in all farms, and the leftovers of the milk were sold to the neighbouring communities. Although in some cases, *S. aureus* was isolated from more than one quarter milk sample of the same cow, each MRSA strain was isolated from a different cow.

A total of 201 (67%) *S. aureus* isolates were recovered from the 300 samples analysed in this study. Overall, 69 MRSA from all farms, were confirmed based on the possession of the *mecA* gene. MRSA contamination was recorded from both clinical and subclinical cases of mastitis (Table 2).

All MRSA were resistant to ampicillin and penicillin followed by trimethoprim (69.6%) and tetracycline (69.6%). All isolates were susceptible to gentamicin followed by vancomycin (95.7%) and ciprofloxacin (91.3%) (Table 3).

Antibiotypes of MRSA circulating in dairy farms included in this study

A total of 13 antibiotypes, designated A1-A13 (Table 4), were identified. The antibiotypes A1, A6 and A8 were identified in all farms while the other antibiotypes were present only in some of the farms.

Possession of virulence factors by MRSA

Phenotypic virulence factor analysis revealed that all the MRSA were hemolytic; 27 isolates (39.1%) were β-hemolytic and 42 isolates (60.9%) were α-hemolytic. All isolates produced caseinase and coagulase. The majority of the MRSA produced lipase (97.1%), gelatinase (94.2%) and lecithinase (91.3%). Genotyping of the MRSA isolates revealed that all MRSA possessed the *coa* gene, followed by 47 (68.1%) for *spa* gene and none for the *tst* and *etb* genes. There was no statistically significant difference between the possession of virulence factors for MRSA strains from subclinical and clinical mastitis for the *spa* gene ($p=0.973$), gelatinase ($p=0.818$), and lecithinase ($p=0.739$).

Discussion

Although milk from cows with clinical mastitis is not expected to be consumed because of its poor quality, the risk of zoonotic transmission is very high for the farmers handling the cows. The presence of MRSA in subclinical mastitis is a major public health concern, especially with the highly prevalent practice of consuming unpasteurized raw milk in the study area [13]. *Staphylococcus aureus* is one of the most common causes of animal disease in dairy farms and a major foodborne pathogen in humans. The contamination of milk with MRSA could be caused by the direct transfer of the bacterial pathogen through mastitis infection of the udder, unhygienic milking process, or contaminated farm environment [24]. The frequent use of antimicrobial agents to treat mammary infections in dairy cows is a risk factor for the emergence of antimicrobial resistance. Milk contaminated with MRSA could be a vehicle for the transmission of

zoonotic pathogens to humans, thereby posing a serious threat to public health [25]. This study is very significant because the data presented here could be used to conduct evidence-based community outreach for awareness and training of dairy farmers, milk handlers and consumers to foster the implementation of hygiene practices in the farms. One of the biggest public health concerns is the potential spread of infectious agents to humans via unpasteurized milk consumption [24].

The contamination of milk with MRSA was recorded in both clinical and subclinical cases of bovine mastitis (24.2% and 20.4%, respectively) in this study. Previous studies in Korea reported lower levels of contamination of mastitic milk with MRSA of 4.3% [26], 13.9% [27] and 2.5% in Southern Italy [28]. While this study reported 67% (201/300) contamination of milk with *S. aureus* from mastitis milk, a recent study also in North West Cameroon reported 48.7% (19/39) [13] from milk regardless of mastitis status. Milk is often contaminated by *Staphylococcus aureus* commonly found on dairy cows [29]. Methicillin-resistant *Staphylococcus aureus* can originate from milk collected from the udder and its spread can be exacerbated by poor sanitation management and contaminated hands of farmers during the milking process [30]. Numerous previous studies have reported different rates of MRSA prevalence on dairy cattle farms in different regions [12, 31, 32]. The variations might be associated with different isolation procedures, sample sizes, sample sources, farm management systems, and other factors [33]. Several studies have reported the presence of MRSA in dairy cattle farms, farm workers, and raw milk, indicating the possible risk of MRSA transmission within and between dairy cattle farms and to the general public [28, 34, 35]. In a recent review that highlighted the role of milk products and milk in the spread of MRSA in the dairy production chain, higher contamination levels were reported for Africa than for Europe and Asia [24]. A recent study by Roy et al. [12] demonstrated a high similarity index (>84%) among MRSA from cows, humans and the environmental surfaces within the same farm vicinity to highlight the zoonotic potential of MRSA and the importance of MRSA control using the 'One Health' approach.

All the MRSA in this study were resistant to penicillin and ampicillin, and this corroborates a previous study that investigated penicillin resistance of staphylococcal isolates from subclinical mastitis in Sohag City, Egypt [36] and another study on raw milk samples from a rural community in Edo State, Nigeria [37]. While susceptibility to gentamicin for MRSA in the present study was 100%, Yang et al. [38] reported that all 73 MRSA were resistant to gentamicin in their study that investigated MRSA from subclinical mastitis in China. An earlier study in Pakistan reported that 10% of the 135 MRSA isolates identified were resistant to gentamicin [39]. We also observed high susceptibility to ciprofloxacin (91.3%). Although this study reported only a 4.3% resistance to vancomycin, a previous study in Cameroon reported high (80%) resistance to vancomycin [13]. This wide difference in the resistance rates for isolates from the same country may be because the isolates come from different regions, and the cattle are reared under different husbandry practices. The emergence of resistance to vancomycin is a feared genetic adaptation in *S. aureus* so far, due to the widespread reliance on this antibiotic for treating MRSA infections in humans [40]. Most isolates (65.2%, 45/69) were multidrug-resistant and this represents a major public health challenge because MRSA carrying antibiotic resistance genes can horizontally transfer these determinants between strains, resulting in pathogen evolution [41].

In this study, we noted that all isolates were α- or β-haemolytic. This finding corroborates the results of Barretti et al. [42]. Alpha-haemolysin has pro-inflammatory and pore-forming properties. It can disrupt the integrity of host cells when it binds to a membrane receptor [43]. Lipase, protease, and lecithinase secretion were detected in most tested strains and this corroborates results from most previous studies [42, 44, 45]. *Staphylococcus aureus* uses *lipolytic and* proteolytic exoenzymes for host tissue invasion, causing damage to the host tissue components and even spreading to other sites [18]. Our isolates lacked the *tst* or *etb* genes and this result corroborates previous studies [46]. The prevalence of the *tst* and *etb* genes from *S. aureus* in cases of bovine mastitis was generally low [46]. However, the study of Zschöck et al. [47] showed a 36.2% prevalence of the *tst* gene in *S. aureus* isolates while that of Teyhoo et al. [48] showed a 14–20% prevalence rate.

Conclusion

This study revealed 23.0% milk contamination with MRSA in the study area. Multidrug resistance was identified in 65.2% of the MRSA isolates, while several isolates possessed virulence factors that can lead to the severity of infection in humans. The presence of resistance and virulence genes suggests a serious risk for transmission to humans through contaminated milk, highlighting the need for better farm hygiene, careful antibiotic use to reduce public health threats and the need for further studies to confirm transmission pathways. We recommend education of herders on measures to minimize contamination and monitoring of multidrug-resistant bacteria in milk and dairy products to prevent the transmission of MRSA from animals to humans.

Limitations

This study did not investigate risk factors associated with MRSA contamination or antimicrobial use in cattle farms. This information is also critical in monitoring and controlling MRSA spread in cattle farms and the local population. Another limitation is that only the *MecA* gene was used to confirm MRSA. Thus the MRSA contamination prevalence reported in this study might be an underestimation. Previous studies have reported that there are alternative gene targets specific to MRSA such as the *blaZ* [49] and the new *MecA* homologue *MecA*(*LGA251*) [50]. Although the data reported here are very pertinent, this is a small-scale study and the findings might not be generalizable to other regions of the country and elsewhere. From the findings reported in this study, we are not able to know if the MRSA strains circulating in the farms are epidemiologically distinct. We, therefore, recommend further studies to identify the MRSA clones circulating in the study area.

Acknowledgements
The authors are grateful to the staff of MINEPIA, North West Region, Cameroon, for assistance in sample collection.

Author contributions
SNE: Data curation, investigation, original draft preparation, review and editing; SFN: Sample collection and investigation, review and editing; LMN: Conceptualization, supervision, resources, review and editing; POB: Conceptualization and resources; NFT: Data curation, review and editing; SIS: Conceptualization, review and editing; RNN: Conceptualization, supervision, resources, data validation, review and editing.

Funding
Partial support was obtained from the South African Medical Research Council through an award to POB. The views expressed here are those of the authors and not necessarily of their affiliated institutions.

Data availability
All data is provided within the manuscript.

Declarations

Ethical approval and consent to participate
This study was reviewed and approved (Ref. No. MINEPIA/DREPIA/NW/40/716/2019) by the Regional Delegation of Livestock, Fisheries and Animal Industries, a subunit of the country's government institution that oversees livestock production and animal health for the North West Region of Cameroon. Collection of the milk samples was done after obtaining informed verbal consent from farm owners. All samples were collected by a trained veterinarian. No invasive procedures were involved, and very little pain was inflicted on the animals during the sample collection process. This study did not involve endangered or protected species and did not use animals for experiments.

Animal ethics declaration
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 14 August 2024 / Accepted: 23 December 2024
Published online: 27 December 2024

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