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Emerging Antimicrobial Resistance in Coagulase-Positive Staphylococci and *E. coli* Isolated from Bovine Clinical Mastitis in Sri Lanka

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MARP and PSF did the contributed for designing, analyzing of the research project. Authors MARP and PSDA contributed for laboratory activity and interpretation of the data. Author MARP wrote the paper. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aim: Mastitis is an economically important disease in dairy cattle, and one of the most common diseases both in developed and developing countries. The disease prevalence is quite high in Sri Lanka; both clinical and subclinical mastitis were reported, *Staphylococcus aureus* and *E. coli* are important bacterial organisms which cause clinical mastitis in dairy cattle. The antimicrobial resistance of these two bacterial species are increasing all around the world, only limited literature is found locally. The objectives of the study were to determine phenotypic antimicrobial-resistant profile in commonly reported two organisms from the clinical mastitis in the country.

Materials and Methods: Samples (n=197) were collected from regional veterinary investigation centers (n = 6) as a routine clinical submission for diagnosis of mastitis, one government dairy farm in 2018-2019. Coagulase positive *Staphylococcus* (n=41) and *E. coli* (n=17) were isolated and identified by conventional tests and standard biochemical tests. Antimicrobial susceptibility testing

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was done and interpreted as described by EUCAST. The coagulase-positive *Staphylococcus* isolates were screened for *mec*A and *E. coli* were screened for CTX-M and NDM by conventional PCR tests as described.

Results and Discussion: Methicillin resistance was not shown in coagulase-positive *Staphylococcus aureus* in the study and a high frequency of resistance was reported against ampicillin (98.0%), amoxicillin (64.7%), amikacin (68.6%), cloxacillin (64.7%) and oxytetracycline (56.9%). *E.coli* were shown 100% resistance to ampicillin, amoxicillin, sulfamethoxazole, and trimethoprim alone. High resistance frequencies were shown for oxytetracycline (80%), chloramphenicol (80%), sulfa trimethoprim combination (66.7%) and doxycycline (60%). Only 26.7% of *E. coli* was shown resistance for cefotaxime and only 4 were shown CTX-M by conventional PCR.

Conclusions: Antimicrobial resistance is emerging in these two species of bacteria, national resistant surveillance and evidence-based antimicrobial usages are recommended to combat against emerging resistance in livestock.

Keywords: Clinical mastitis; Staphylococcus; E. coli; antimicrobial resistance; Sri Lanka.

1. INTRODUCTION

Mastitis is an economically important disease in dairy farms all over the world [1,2]. It is a widely spread disease, clinical mastitis and subclinical mastitis are equally significant with direct and indirect losses in the dairy industry [3]. In Sri Lanka, the disease prevalence was 40-50% among dairy cattle [2]. Over 11 000 clinical cases were reported in 2014 island-wide [4]. Mastitis is costly due to the reducing milk production, changing the quality of milk, milk rejection, high treatment cost, premature slaughtering and culling or death of animals [5]. The public health importance should not underestimate due to the shedding of toxins, contamination of bacterial organisms, and antimicrobial residues in milk Mastitis is still considered as one of the challenging diseases in the global dairy industry [6].

Based on the clinical classification, mastitis have been described in the literature as clinical and subclinical mastitis [2]. Although subclinical mastitis is common, the burden of clinical mastitis is high with clinical signs together with abnormalities in milk [2]. Bacteria are one of the main causative organisms together with fungus and viral or mycoplasma organisms [7]. The frequent pathogens in cows were *Staphylococcus aureus*, *Streptococcus* spp., *Escherichia coli, Pseudomonas* spp., and *Mycoplasma* spp. [7]. Although more than 150-microorganism have been found in the infected mammary gland, both contagious organisms such as *Staphylococcus* and environmental contaminants such as *E. coli* is equally important in the local dairy industry [2]. In many studies, improved management practices improve both types of clinical mastitis in cattle [8]. However, clinical mastitis, which caused by coagulase-positive *Staphylococcus* and *E. coli* is equally important in the mid-country and upcountry in Sri Lanka [2]. *Staphylococcus* and *E. coli* were reported among frequent isolates in previous studies published together with *Klebsiella*, *Streptococcus* [4,9]. In addition, only a few numbers of studies on clinical mastitis published in Sri Lanka although significant improvement expected in the recent past. However, risk factors for clinical mastitis have been identified in a number of studies published including both management and hygienic practices [10,11].

Antimicrobial resistance is an emerging problem in the dairy industry and resistant phenotypes have been encountered in milk [7]. Methicillin-resistant *Staphylococcus aureus* has been isolated in milk from clinical mastitis in India previously, potential risk is there with similar management practices in the subcontinent [12]. Similarly, both New Delhi metallo-beta-lactamase (NDM) and Extended spectrum of beta lactamases (ESBL) have been also reported in milk from clinical mastitis in cows although no reports were found locally [13]. NDM and ESBL resistant *E.coli* were often shown either multi drug resistant or co resistant to other antimicrobials. Furthermore, NDM is endemic in India and South Asia, it is considered as worth of exploring NDM in among clinical isolates. All these beta lactamases such as ESBL, NDM inactivate cephalosporin and carbapenem respectively. Non-empiric treatment with a wide range of antimicrobials is widely practiced for mastitis in the local dairy industry. CTX-M 15 has been reported common in milk from clinical mastitis previously. The veterinary consultation

regarding clinical mastitis of dairy cows is limited and veterinarians are consulted only for severe clinical conditions or systemic infection in the country. The bacterial isolation and identification before the treatment for mastitis are limited among small-scale farmers due to several practical difficulties and other unknown reasons. Although evidence-based antimicrobial usage is highly recommended in regional veterinary laboratories, attitude of veterinarians and livestock farmers has not been changed or improved. Both categories may rely on previous experiences of treatment or management of clinical mastitis. Therefore, the study determined phenotypic antimicrobial resistance profiles in commonly reported two organisms from the clinical mastitis in local herds in the country.

2. MATERIALS AND METHODS

The study was done in (January to December) 2018 at the Veterinary Research Institute (VRI). The clinical submission of milk samples was received from six regional laboratories and a large-scale dairy farm (Altogether n = 7). The milk samples were transported under 4^0C when each sample was stored in -18^0C just after collection. The submitted milk samples were cultured on 5% sheep blood agar (Oxoid) and incubated overnight at 37^0C. Sub-culturing was done the following day into 5% sheep blood agar (Oxoid) and all isolates were preserved and stored at -4^0C until usage. The isolates were identified by examining cultural morphology, Gram staining, and biochemical tests (catalase test, oxidase test, SIM, TSI, urease, tube coagulase test, citrate, indole, sugar fermentation and hyaluronidase test) and only coagulase-positive *Staphylococcus* and *E. coli* were selected for the future studies as described previously [14].

The pure cultures of coagulase-positive *Staphylococcus* and *E. coli* were cultured on 5% sheep blood agar and antimicrobial susceptibility tests/disk diffusion test was done and interpreted as described by EUCAST (www.eucast.org). Antimicrobial susceptibility tests were done for following antimicrobials such as ampicillin (10 µg), amoxicillin (25 µg), amoxicillin & clavulanic acids (20 µg & 10 µg), cefoxitin (10 µg), ciprofloxacin (5 µg), sulfa trimethoprim (23.75-1.25 µg), amikacin (30 µg), kanamycin (30 µg), tetracycline (30 µg), neomycin (30 µg), streptomycin (10 µg), chloramphenicol (30 µg), cefotaxime (30 µg), cloxacillin (30 µg) meropenem (10 µg), gentamicin (10 µg) and doxycycline (30 µg). The *mec*A gene was screened only in cefoxitin-resistant coagulase-positive *Staphylococcus* as described by Name [15]. CTX-M gene was screened using universal primers as described by Bialvaei et al. [16]. The isolates which were shown resistant to meropenem were screened for NDM as described previously [17].

3. RESULTS

Altogether 197 milk samples were received from clinical mastitis cases from six regional laboratories and one big dairy farm. Of all samples were cultured and incubated at 37^0 for 24-48 hours; however, 23.8% (n = 41) of them did not show growth on 5% sheep blood agar. Coagulase positive, catalase-positive, oxidase negative, urease positive, hyaluronidase positive, Gram positive cocci were taken as *Staphylococcus aureus* in the study. Catalase positive, oxidase negative, citrate negative, urease negative, H_2S negative, motile, gas positive and indole positive, Gram negative rods were identified as *E. coli*. Both coagulase positive *Staphylococcus* (n = 51) and *E. coli* (n = 17) were recovered in the study at the submitted clinical submissions. As it is shown in Table 1, a high frequency of *Staphylococcus* isolates resistance was reported against ampicillin (98.0%), amoxicillin (64.7%), amikacin (68.6%), cloxacillin (64.7%) and oxytetracycline (56.9%). Over 50% of *Staphylococcus* isolates were multi drug resistant in the study. The *mec*A gene was not detected in five coagulase-positive isolates those were phenotypically resistant to cefoxitin. *E. coli* were shown 100% resistance to ampicillin, amoxicillin, sulfamethoxazole, and trimethoprim alone. High resistance frequencies were shown for oxytetracycline (80%), chloramphenicol (80%), sulfa trimethoprim combination (66.7%) and doxycycline (60%) (Table 2). CTX gene was observed only in 4 isolates of *E. coli* and further sequencing of PCR products was not done due to the unavailability of sequencing and funding. Either MRSA or NDM was not isolated from coagulase-positive *Staphylococcus* and *E. coli,* respectively. Only two *E. coli* isolates were shown phenotypic resistance to meropenem in the study from the mastitis clinical submission.

4. DISCUSSION

Coagulase positive Staphylococcus was isolated from slightly one-fourth (25.9%) of clinical submission while *E. coli* was isolated and identified in 8.6% in the study. In the literature,

Staphylococcus and *E. coli* were isolated in 80% and 14% of cow mastitis in Sri Lanka, respectively [18]. Impact, the current study is the largest collection of isolates from clinical mastitis in the country although a number of studies published with the limited number of samples of mastitis. The percentage of isolation of coagulase positive Staphylococcus was low in our study for unknown reason and 23.8% of samples were not shown any bacterial growth. Unknown usage of antimicrobials either by parental or intra mammary route before the sampling might be a possible explanation for that kind of results.

No MRSA was isolated from single sample received in the study. Although we expected more in numbers, New Delhi metallo-beta-lactamase (NDM) were not found in this study from clinical mastitis in cows. Both MRSA and NDM are often multi drug resistant, further lababrotry conformation are required. Conversely, cow's milk was identified as a source of MRSA infection in humans either by consumption or handling of contaminated milk [12]. The percentages of MRSA were varied in 1-13% of milk in India, while zoonotic transmission were also reported [19]. Furthermore, handling of milk and milk consumption pattern is different in these two countries in many ways although geographically located closely. Multidrug resistance have been also reported in more than 50% coagulase positive *Staphylococcus* which indicates risk of repeat antimicrobial therapy. Notably, MRSA is often multidrug resistant, and selection of an appropriate antimicrobial is challenging on therapeutic purpose. MRSA is an endemic bacterial type in Sri Lanka and frequency of determination were varied in 9-85% percentage in human while few reports were found in the field of livestock [20-22]. In contrast, no report was published in milk as a source of human MRSA infection in the local context. Out of limited collection of literature in livestock, 10% and 26% of pig farmers were reported positive for MRSA while no isolates were found in pigs [20,21]. In addition, livestock associated Methicillin-resistant Staphylococcus aureus (LA MRSA) such as ST398, ST9 sequence types have not been reported in Sri Lanka [20]. In global context, a high percentage of MRSA in livestock was reported in China as 47.6% [9].

Among coagulase-positive *Staphylococcus*, is more than 50% resistant to β-lactams, trimethoprim, aminoglycoside, tetracycline, cloxacillin, neomycin and amikacin. These antimicrobials are used common in the country for therapeutic purposes and dry cow therapy. Importantly, β-lactams (cephaperin benzathine, ampicillin and cloxacillin) are used in large quantity as dry cow therapy in Sri Lanka. In addition, 35% and 45% of isolates of *Staphylococcus* were observed resistant to sulfamethoxazole and chloramphenicol, respectively. None of isolates were resistant to ciprofloxacin in the study. Ciprofloxacin and chloramphenicol are banned in livestock and they are only allowed to be used for human purpose. However, enrofloxacin is used widely in dairy. Enrofloxacin is added into the locally made intramammary infusion at regional laboratories although methodology is not encouraged by clinical microbiologist. In addition, parenteral administration of β-lactams, sulfamethoxazole and enrofloxacin are common in large animal veterinary practices. Furthermore, phenotypic antimicrobial resistance in *Staphylococcus* isolates from similar capacity were observed low as 30% in other developing countries such as Egypt [23].

However, high percentage of phenotypic resistance (100%) were reported against penicillin, nalidixic acid in *Staphylococcus aureus* under similar condition, Ethiopia [24]. These finding were suggested of differences in phenotypic antimicrobial resistance in country to country.

E. coli isolates were 100% resistant to β-lactams, sulfamethoxazole, trimethoprim and over 80% of them to chloramphenicol and tetracycline. As mentioned previously, β-lactams, tetracycline and enrofloxacin are commonly used both for intramammary infusion and parenteral injection in dairy practice [25]. In addition, over 50% of resistance was observed for aminoglycoside, sulfa trimethoprim. Gentamicin and streptomycin were banned in the livestock practiced for long time in Sri Lanka. Importantly, only two isolates were phenotypically resistance for meropenem while NDM gene was not detected. Other carbapenem resistance were not examined in the study such as SHV or OXA.

Overall, a high trend of phenotypic antimicrobial resistance was observed on commonly used agents. Non-empirical antimicrobial treatment is not encouraged on repeating clinical mastitis cases although several practical difficulties are existed in this area. The field veterinarians have limited time in participate in clinical cases and urgent antimicrobial are required for acute and

acute systemic mastitis in cows. The long time required is considered as a limiting factor and minimum 72 hours are required to complete disk diffusion test. In practice, cow owners do not hesitate to apply antimicrobials without veterinary consultation. Although regional laboratory is equipped with basic infrastructure to carry out antimicrobial susceptibility testing, owners prefer non-empiric treatments without further confirmation.

The risk factor associated with clinical mastitis in Sri Lanka has been studied by Gunawardana et al. [26]. Unhygienic environments, inadequate knowledge or practice of mastitis control, animal-related factors such as parity, milk yield, management practices such as milking practices, access to veterinary services, use of veterinary products, stall structure, and stall hygiene were identified as the most relevant risk factors. Most of these factors can be minimized by good husbandry practice in dairy farming.

Table 1. Phenotypic antimicrobial resistance in coagulase positive *Staphylococcus* in milk from clinical mastitis sample (n = 51)

Antimicrobial agent	Number of resistant phenotypes	% of Resistant phenotypes
Ampicillin	50	98.03
Amoxicillin	33	64.70
Cefoxitin	5	9.8
Ciprofloxacin	0	0
Sulfa trimethoprim	18	35.29
Trimethoprim	27	52.90
Amikacin	35	68.62
Kanamycin	22	43.13
Oxytetracycline	29	56.86
Doxycycline	18	35.29
Neomycin	28	54.90
Chloramphenicol	23	45.09
Cloxacillin	33	64.70

Table 2. Phenotypic antimicrobial resistance in *E. coli* in milk from clinical mastitis sample (n=15)

Antimicrobial agent	Number of resistant phenotypes	% of Resistant phenotypes
Ampicillin	15	100.00
Amoxicillin	15	100.00
Amoxicillin + clavulanic acid	4	26.67
Gentamicin	7	46.66
Neomycin	9	60.00
Cefotaxime	4	26.67
Ciprofloxacillin	4	26.67
Trimethoprim	15	100.00
Sulfamethoxazole	15	100.00
Sulfa trimethoprim	10	66.67
Amikacin	3	20.00
Chloramphenicol	12	80.00
Oxytetracycline	12	80.00
Doxycycline	9	60.00
Streptomycin	6	40.00
Meropenem	2	13.33

5. CONCLUSION

MRSA, and carbapenem resistance with genetic evidence were not found in milk from studied clinical mastitis cases. However, a high percentage of phenotypic antimicrobial resistance was observed in Coagulase positive *Staphylococcus* and *E. coli* to both commonly used antimicrobials in cattle in Sri Lanka. Therefore, evidence-based selection of antimicrobials is encouraged to minimize emerging resistance of bacteria in milk from mastitis animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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