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Antimicrobial Resistance of Pathogenic Bacteria Isolated from Mastitis Cows in Khartoum State, Sudan

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Authors' contributions

This work was carried out in collaboration between all authors. Author YAS designed the study, wrote the protocol and supervised the laboratory work. Author WMY carried out the laboratory work, wrote the first draft of the manuscript and managed the literature searches. Author AAEG revised the manuscript. Author MEM performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The problem of antimicrobial resistance (AMR) is now recognized as a major threat to the health and development in all countries. This study was conducted to determine pathogenic bacteria associated with clinical cases of bovine mastitis and their antimicrobial resistance patterns.
Study Design: This study was carried out In the Department of Bacteriology, Central Veterinary Research Laboratory, Khartoum, Sudan during the period from April 2013 to March 2014.
Methodology: 150 milk samples from clinical cases of bovine mastitis were cultured onto blood agar plates and the isolated organisms were identified by conventional bacteriological methods. One hundred and five isolates were tested against 11 antimicrobial agents commonly used in the dairy farms using the disc diffusion method.

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Results: The majority of the isolates were highly sensitive to gentamycin, ciprofloxacin, norfloxacin and kanamycin, highly resistance to penicillin-G and moderately sensitive to novobiocin, tetracycline and cefalexin.
Conclusions: This study revealed that a number of significantly public health concern bacteria were isolated from milk samples and increasingly developed resistance to different groups of antimicrobial agents.

Keywords: Bacteria; pathogenic; cows; mastitis; identification; antibiotics; resistance; Khartoum.

1. LITRATURE REVIEW

Antimicrobial resistance (AMR) is an increasingly serious threat to global public health. As a part of the response to this global problem, World Health Organization (WHO), indorsed a Global Action Plan on AMR (GAP) which was approved by the World Health Assembly in 2015 [1]. In accordance to one health approach WHO, Food Agriculture Organization (FAO) and International Epizootics Organization (OIE) committed to implement the Global Action Plan on antimicrobial resistance targeting main bacteria that increasingly resistant to antibiotics among which are *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pneumonia* and *Salmonella* spp*.* Drug resistance emerges only when the antibiotic or antimicrobial drug and the genetic resistance determinant in microorganisms selected by the antimicrobial drug come together in an environment or host, which can lead to many clinical problems [2,3]. Millions of kilograms of antimicrobials are used every year in the prophylaxis and treatment of people, animals and agriculture globally, driving the resistance problem by killing susceptible strains and selecting those that are resistant [4,5,6]. Methicillin resistance in *S. aureus* was initially detected in Europe in the 1960s shortly after the introduction of methicillin [7]. A cost comparison of treating methicillin-resistant (MRSA) versus methicillin susceptible (MSSA) *S. aureus* in New York City found almost a threefold increase in mortality (21% versus 8%) and an economic cost increase of 22% associated with MRSA. Enteric organisms such as *Salmonella, Campylobacter, Listeria*, *Enterococcus* and some strains of *E. coli* are propagated primarily among animals and subsequently infect people. The transfer may occur through the food chain or through animal handlers [8,9]. It was reported that the common urinary pathogens in the Sudan; *E. coli, K. pneumoniae,* and *Proteus mirabilis* have showed high rates of resistance to ampicillin, amoxicillin, co-trimoxazole, tetracycline, sulfonamide, trimethoprim, streptomycin, and carbenicillin [10]. Previous studies have indicated that the overuse of antibiotics in human and veterinary medicine have a role in the development of antibiotic-resistance strains [11]. On the other hand, some investigators have reported that animal contribution to the resistance problem in human infections is small but not insignificant; they have a major role if enteric organisms are involved [12]. The present study was conducted to evaluate the antimicrobial resistance pattern of bacteria isolated from clinical cases of bovine mastitis in Khartoum State, Sudan.

2. MATERIALS AND METHODS

2.1 Collection of Milk Samples

A total of 150 milk samples were collected aseptically from local and cross-breed dairy cattle farms from three localities in Khartoum State, Sudan. The teats were cleaned with a cotton piece impregnated in 70% alcohol. 20 to 25 ml of the milk secretion was collected in sterile plastic containers. All samples were placed in ice and immediately transported to the Department of Bacteriology, Veterinary Research Institute (VRI) and used in the same day for bacteriological analysis.

Each undiluted milk sample was streaked onto 5% sheep blood agar plates (Oxoid, CM 271, UK) using sterile cotton swab and incubated aerobically at 37℃ for 24 hours in an aerobic incubator (Scott Science, Model LIB 080M, serial no. 08101705, UK). The incubation was further continued to 48 hours if no growth was observed after 24 hr. before discarded as negative for growth.

2.2 Isolation and Preservation of the Isolates

Well isolated representatives of the bacterial colonies were selected and subcultured onto blood agar and nutrient agar plates (Oxoid, CM

1, UK) then incubated at 37℃ for 24 hours. The obtained pure cultures were further subcultured on blood agar slant, incubated at 37℃ for 24 h. then stored in a refrigerator (Coldair, Model H.P, Serial no. 06-207538, Sudan) at 4℃ for further analysis.

2.3 Identification of the Isolates

The purified isolates were identified by conventional bacteriological methods [13,14], including Gram staining reaction, aerobic and anaerobic growth, motility test, catalase activity, oxidase test acid from glucose and oxidation-fermentation (OF) tests, urease activity, indole production, nitrate reduction, H_2S production from triple sugar iron agar (TSI), citrate utilization, methyl red (MR) and Vogues-Proskaür (VP) tests. In addition to growth in medium supplied with 6% and 10% (w/v) sodium chloride, coagulase test for *Staphylococcus* spp., and carbohydrates (sucrose, maltose, lactose, manitol, raffinose, fructose, xylose and glucose) breakdown in peptone water medium were also analyzed.

2.4 Antibiotic Sensitivity Testing

Antibiotic sensitivity testing was performed for the identified isolates using the modified disc diffusion technique (Kirby-Bauer 1960). Two to four well isolated colonies from fresh culture of the bacterium under test were picked up with a wire loop and mixed in 5 ml 0.85% sodium chloride (LOBA CHEMIE, Art.5819, India) in a test tube, adjusted to the turbidity of McFarland tube No. 0.5 and incubated at 37ºC for two hours. Muller-Hinton agar (Micro master, DM172, India) plates were removed from the refrigerator and dried in the incubator at 37℃. A sterile non–toxic swab was dipped into the suspension; the excess fluid was removed by pressing the swab against the edges of the tube. The swab was streaked over the entire surface of two Muller-Hinton agar plates at three different directions. The plates were allowed to dry, then each single antibiotic disc (UNIPATH LIMITED, Basingstoke, and Hampshire, England) (Table 1) was picked by sterile forceps and placed on the surface of the streaked medium and pressed gently to ensure full contact with the surface of the agar. The plates were incubated at 35℃ for 24 hours. That obtained inhibition zones diameters were measured by transparent rules at the back of the plate. The results were recorded as sensitive (S), intermediate (I) or resistance (R) according to zone size in the interpretation chart [14].

3. RESULTS

One hundred twenty six bacteria were isolated from 150 milk samples, including 100 (79.4%) Gram positive, 23 (18.3%) Gram negative bacteria and 3 (2.4%) yeasts. *Staphylococcus* spp. was the most isolated bacteria 67(53.2%), followed by *Streptococcus* spp. 22(17.5%) and the least isolated bacteria were *E. coli*, and 1 (0.7%) of each of *Actinomyces* sp., *Pseudomonas* sp., *Proteus mirabilis* and *Enterobacter* sp. and 3(2.4%) yeasts (Table 2).

Table 1. Antimicrobial agents used for susceptibility tests

Antimicrobial agent	Code	Concentration
Amoxicillin	AMX 10	10 µg
Ciprofloxacin	Cap 5	5 µg
Tetracycline	T 30	30 µg
Gentamycin	Gen 10	10 µg
Penicillin –G	P 10	10 unit/ disk
Kanamycin	K 30	30 µg
Norfloxacin	NX 10	10 µg
Novobiocin	NV 30	30 µg
Lincomycin	MY 10	10 µg
Cefalexin	CN 30	5 µg
Streptomycin	S 10	10 µg

Table 2. Frequency of bacterial isolates

No.	Organisms	Frequency
1	*Staphylococcus* spp.	67(53.2%)
2	*Streptococcus* spp.	22(17.5%)
3	*Enterobacter* spp.	14(11.1%)
4	*Corynebacterium* spp.	6(4.8%)
5	*Klebsiella pneumoniae*	5(4.0%)
6	*Bacillus* spp.	4(3.2%)
7	*Yeasts*	3(2.4%)
8	*E. coli*	2(1.6%)
9	*Actinomyces bovid*	1(0.8%)
10	*Pseudomonas aeruginosa*	1(0.8%)
11	*Proteus mirabilis*	1(0.8%)
	Total	126(100%)

Most of the tested organisms were highly sensitive to ciprofloxacin, norfloxacin, gentamycin and streptomycin which are the most effective drugs, while the resistance was more frequent to penicillin G, amoxicillin, lincomycin, cefalexin, and then tetracycline which were significant at $p < 0.001$ (Table 3 and Fig. 1). In addition, based on the results of this study (Table 3 and Fig. 1), ciprofloxacin and novobiocin were recommended for treatment of bovine mastitis, while penicillin was not recommended. Table 4 represents the analysis of the variance.

Table 3. Antibiotic sensitivity testing results

Antibiotics	Pattern of tested bacteria reaction			Total of tested organisms
	S	R	I	
Ciprofloxacin	95(95%)	02(2%)	03(3%)	100
Gentamycin	102(99.03%)	01(0.97%)	0(0%)	103
Norfloxacin	101(98.05%)	02(1.95%)	0(0.0%)	103
Kanamycin	84(81%)	11(10.5%)	10(9.5%)	105
Streptomycin	82(83.7%)	10(10.2%)	06(6.12%)	98
Novobiocin	72(69.2%)	24(23.1%)	08((7.7)	104
Tetracycline	63(60%)	24(22.9%)	18(17.1%)	105
Cefalexin	66(63.4%)	30(28.8%)	08(7.7)	104
Lincomycin	54(60.4%)	46(43.8%)	05(4.77%)	105
Amoxicillin	45(44.6%)	54(53.5%)	21.98%)	101
Penicillin –G	13(12.5%)	84(80.77%)	07(6.7%)	104

Key: (S) Sensitive, (I) Intermediate or (R) Resistance

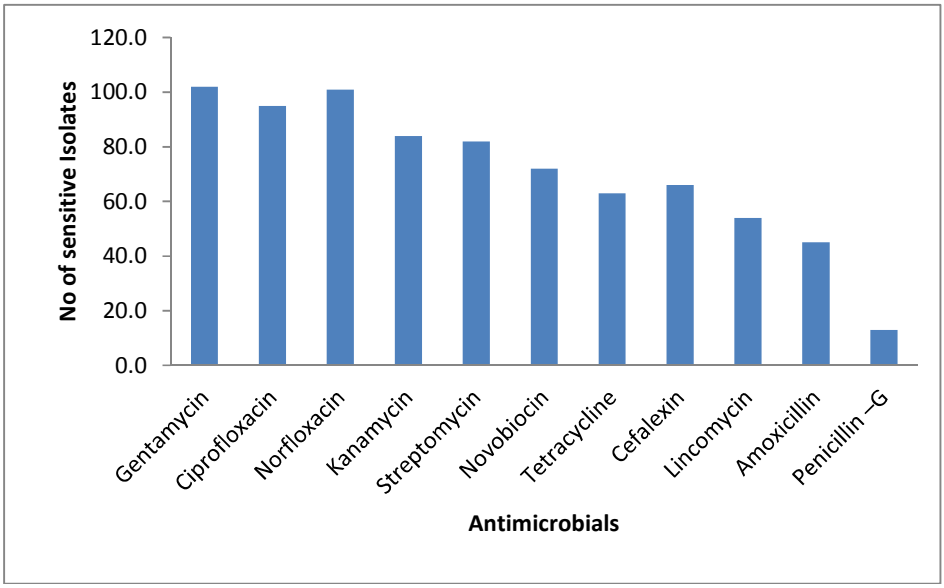

Fig. 1. Number of sensitive isolates

Table 4. Analysis of variance (ANOVA) for antibiotics with its sensitivity tests

Sensitivity test	Sum of squares	df	Mean square	F	Sig.
Between groups	102.806	10	10.281	27.223	.000
Within groups	425.606	1127	.378		
Total	528.412	1137			

4. DISCUSSION

Antibiotics continued to have an effective role in the treatment of infectious diseases and have significantly contributed to the reduction in morbidity and mortality associated with infectious diseases. Antimicrobial drug resistance in both human and veterinary medicine constitutes a major problem in industrialized and resource-poor countries. Since the introduction of antibiotics into clinical use in the mid-1940s, microorganisms have shown a remarkable ability to protect themselves by developing and acquiring antibiotic resistance. It was reported that the overuse or misuse of antibiotics has been linked to the emergence and spread of micro-organisms that are resistant to them, rendering treatment ineffective and posing a serious risk to public health [15]. Many investigators have outlined the relationship

between antibiotic resistance in humans and resistance in animals, with the conclusions varying widely from minimal impact to a substantial impact [16]. In this study, ciprofloxacin, norfloxacin, gentamycin and streptomycin were found to be more effective against all the tested bacteria, similar to the previous report [17]. On the other hand, the isolates were more resistant to penicillin, amoxicillin and tetracycline which was in agreement with the results of other investigators [18,19].

K. pneumoniae isolated in this study is a common cause of Gram-negative urinary, respiratory tract blood stream and occasionally associated with bovine mastitis. Resistance of *K. pneumoniae* to the third-generation antibiotics cephalosporins is a public health concern and increased significantly in several countries as well as resistance to carbapenems in recent years. *E. coli* is one of the most common food-borne pathogens worldwide. Antimicrobial resistance in *E. coli* continues to increase throughout Europe [20]. Methicillin-resistant *S. aureus* (MRSA) is one of the most important causes of antibiotic-resistant healthcare-associated infections worldwide. In the United States and the United Kingdom, 40–60% of nosocomial *S. aureus* strains are MRSA [21]. Some studies in the USA estimated that 300 million people are expected to die prematurely because of drug resistance over the next 35 years, and the world's GDP will be 2 to 3.5% lower than it otherwise would be in 2050. From now on, up to 2050 the world will lose 60-100 trillion USD of economic output if antimicrobial drug resistance is not tackled [22]. It is still remains a significant public health problem, as the percentage of MRSA of all invasive *S. aureus* infections (bloodstream and cerebrospinal fluid) is above 25% in eight out of 28 countries, mainly in Southern and Eastern Europe [20]. Previous studies had showed that the long-term use of a single antibiotic will select for bacteria that are resistant not only to that antibiotic, but to several others [23].

5. CONCLUSION

In this study a number of bacteria isolated from mastitis cows were found to be multidrug resistant. Gentamycine and ciprofloxacine showed high efficiency against the tested organisms and were recommended for the treatment of bovine mastitis while gentamycine is of no value for the treatment of the disease.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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