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Frequencies and Antibiotic Susceptibility Patterns of Bacteria Causing Mastitis among Cows and Their Environment in Khartoum State*

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Abstract: The present study was conducted to investigate mastitis causing organisms in dairy cows and to correlate them with the isolates from udder skin of cows as well as their environment (air). The data revealed higher incidences of subclinical mastitis than the clinical form of the disease. *Staphylococcus aureus* (11.5%), *Streptococcus agalactiae* (0.83%), *Escherichia coli* (7%), *Klebsiella pneumoniae* (9.4%) and *Enterococcus faecium* (2.5%), were the important pathogens isolated from mastitic cows' milk samples. The udder' skin of the cows revealed the presence of *Pseudomonas aeruginosa* (9.5%) and *Staphylococcus aureus* (0.95%). However, the samples sediment from the air of the cows fences, revealed as a pathogen, only the presence of *S. aureus* (0.95%). Other non- serious mastitis pathogens were also found, in the three groups of samples, during the present study. Antibiotic susceptibility tests were undertaken. The most prominent resistant was those of *S. aureus* towards tetracycline (79.41%) followed by penicillin (29.41%) and amoxacillin- cluvenic acids (20.59%). The susceptibility of *E. coli* showed resistance towards erythromycin (76.47%), tetracycline (29.41%), streptomycin and sulfamexazol-trimethoprim (17.65%) and kanamycin (5.88%). Similarly *Enterococcus faecium*, showed multiple range of resistant to the drugs tested.

Key words: Mastitis pathogens, antibiotic susceptibility, incidence, cows, Sudan

Introduction

Mastitis is the inflammation of the mammary glands. It is caused by the invasion of the quarter, usually, via the teat canal, with a variety of microorganisms in all species of mammals (Hunter, 1994). Bovine mastitis is a complex disease; it involves the three-bio systems; the microorganisms, the environment and the host (the cow); (Heeschen, 1984). Moreover, the rates of clinical mastitis and severity of clinical signs differed among the herds, seasons of the year, parity groups and stages of lactation (Hogan *et al.*, 1989).

Bartlett *et al.* (1992) reported an increase prevalence of coliform and environmental streptococcal infection, due to improper management. Moreover, Kamata *et al.* (1990) identified 54.7% of coagulase-negative Staphylococci, *Corynebacterium* sp., Gram-negative bacteria and *S. aureus* in 30.2, 28.5 and 25% were isolated as mastitis pathogens respectively, by the same author. They also could isolate

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Streptococcus sp. (*Streptococcus agalactiae*, *St. dysgalactiae*, *St. uberis* and *St. bovis*) in about 33.7%. In another trial Bezek (1998) determined 15.3% coliforms, 27.1% *Staphylococcus* sp. and 26.6% *Streptococcus* sp., while, Sargeant *et al.* (1998) isolated *Staphylococcus aureus* (6.7%), *Streptococcus agalactiae* (0.7%), other *Streptococcus* sp. (14.1%), coliforms (17.2%), Gram-positive Bacilli (5.5%), *Corynebacterium bovis* (1.7%) and other *Staphylococcus* sp. (28.7%). Similarly, Sobiraj *et al.* (1997) in 35.3% of bacteriological positive milk samples could detect *S. aureus*, Enterococci, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae* in 8.9, 8.2, 8.1 and 4.9%, respectively, for all positive milk samples. Similarly Kudinha and Simango (2002) reported that in Zimbabwe, the most frequent pathogens isolated in clinical mastitis were the enteric bacteria (31.4%), followed by coagulase negative staphylococci (22.9%) and then *Staphylococcus aureus* (17.1%), whereas in subclinical mastitis *S. aureus* (34.2%) and coagulase-negative staphylococci were (33.2%) the most common. Bacillus species were only isolated in milk samples from subclinical mastitis.

The lack of appropriate mastitis therapy results in the development of resistant organisms to antibiotics (Linhart and Weiskopf, 1989), especially in improperly treated cows (Jonsen and Eberhart, 1981; Robinson *et al.*, 1988). Moreover, use, misuse and often abuse of antimicrobial agents has encouraged the evolution of bacteria towards resistance, resulting into therapeutic failure (Straut *et al.*, 1995). Furthermore resistance can be transferred between species of different genera (MAFF, 1998). The highest resistance was to tetracycline (17.6%), followed by lincomycin (13.7%). About 8% of the isolates were resistant to both penicillin and streptomycin (Kudinha and Simango, 2002).

The aim of the present study was to investigate the occurrence of mastitis incidences, investigating of the causative agents of mastitis, to test their susceptibilities to antibiotics and to correlate the isolates from udder skin of the cows and from their environments (air samples) in dairy cows in Khartoum State.

Materials and Methods

Farms

The data for the present study was collected during July to September 1998 on Sudanese dairy cows from Khartoum State (Sudan), while the laboratory work was done at Berlin, Germany during October 1998 to March 1999. Dairy land farm (Friesian cows, closed system), from Khartoum and other herds from Khartoum North and Omdurman (local and cross breeds, open systems) were selected for the present study.

Diagnosis of Mastitis

California Mastitis Test (CMT) was done in the selected dairy farms. Physical examinations were also conducted on all CMT-positive cows. Microbiological examinations were carried out in all mastitis-suspected milk samples.

Incidences of Mastitis in the Quarters of the Examined Cows

According to CMT results, the quarters of each diseased cow were divided into clinically infected (subdivided as watery, hemorrhagic or purulent), subclinically infected and healthy quarters. Dry quarters, mostly as a result of previous infections, were calculated. Similarly, the rates of infection in the quarters, depending on its location, were also estimated.

Isolation and Identification of Mastitis Causing Agents

Collection of Milk Samples

Aseptic quarter milk samples from suspected cases were collected into sterile bottles. Swaps were also collected from cow's udder skin aseptically; Manitol salt agar plates were open at different sites of the farms for sedimentation of pathogenic Micrococci. All milk samples were cooled and transferred to the laboratory in an ice-box (-4+0°C).

Identification of the Organisms

Isolation and identification of the collected bacteria, were carried out according to Cowan and Steel's manual (Barrow and Feltham, 1993), the difficult cases were done after Berg Manuals (Holt *et al.*, 1994). A loop (50 µL) of the sample was streaked on a quadrant of both a blood and MacConkey agar plates. After incubation (24-48 h, 37°C), mastitis-suspect colonies were picked up, subcultured and identified according to their morphological, biochemical and serological properties. *Staphylococcus aureus*, *E. coli* and *Streptococcus* sp. were identified with the API 20Staph, API 20E and API 20Strep, respectively, according to the manufacturer's instructions (Bio Merieux, France).

Antibiotic Susceptibility Tests

Mastitis causing pathogens were examined for their resistant patterns by the agar diffusion assay (German DIN standard), similarly, the NCCLS standard and the manufacturer values, were used, for antibiotics which were not found in DIN standard. The following antibiotics were selected to test the susceptibilities of the isolates (enrofloxacin 5, naldixic acid 30, oxacillin 5, erythromycin 15, streptomycin 25, kanamycin 30, gentamycin 10, lincomycin 15, neomycin 30, penicillin 6, amoxicillin-clavulanic acids 20+10, sulfamexazole+ trimethoprim 23.75+1.25 and tetracycline 30 µg). Zones of growth inhibition around the antibiotic disks were evaluated after over night incubation on Muller-Hinton agar at 37°C as sensitive, intermediately sensitive or resistant.

Statistical Analysis

The incidence of infected quarters and the antibiotics susceptibility tests were calculated as a percentage of the total number of the examined quarters and strains of bacteria isolated, respectively.

Results

Isolation and Identification of Mastitis Causing Bacteria

Isolates from Mastitis Milk

The frequency of the isolation of mastitis causing bacteria (Table 1) showed that *S. aureus* (29 = 11.9%) were the most predominant pathogens identified, followed by *K. pneumoniae* (23 = 9.43%) and *E. coli* (17 = 6.7%). However, few isolates (2 = 0.82) of *Streptococcus agalactiae* were found in one cow. Other isolated organisms were *Bacillus cereus* (2 = 1.23%), *Enterococcus faecium* (6 = 2.46%), *Arcanobacterium pyogenes* (1 = 0.41%), *Pasteurella multocida*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* (2 = 0.82%). Moreover, high incidence (37 = 15.16%) of *Bacillus* sp. and few isolates of *Streptococcus* sp. (2 = 0.82%) and *Enterococcus* sp. (1 = 0.41%) were also found.

Table 1: Isolates from different sources of mastitic cows in dairy farms at Khartoum State

Isolates	Milk	Udder skin	Air
<i>B. cereus</i>	3 (1.23%)	1(0.95%)	0
<i>Streptococcus agalactiae</i>	2 (0.823%)	0	0
<i>Enterococcus faecium</i>	6 (2.46%)	0	0
<i>Staphylococcus aureus</i>	29 (11.9%)	1 (0.95%)	5 (2.7%)
<i>Arcanobacterium pyogenes</i>	1 (0.41%)	0	0
<i>Escherichia coli</i>	17 (6.7%)	0	0
<i>K. pneumoniae</i>	23 (9.43%)	0	0
<i>P. multocida</i>	2 (0.823%)	0	0
<i>P. aerogenosa</i>	1 (0.41%)	10 (9.5%)	0
<i>A. hydrophila</i>	2 (0.823%)	0	0
<i>R. equi</i>	0	1 (0.95%)	0
<i>Bacillus</i> sp.	37 (15.16%)	14 (14.3%)	51 (27.4%)
<i>Streptococcus</i> sp.	1 (0.41%)	0	2 (1.08%)
<i>Enterococcus</i> sp.	1 (0.41%)	0	0
<i>Proteus</i> sp.	0	3 (2.85%)	0
Alcaligenes	0	1 (0.95%)	0
Micrococcaceae	32 (13.11%)	0	27 (14.5%)
Enterobacteriaceae	18 (7.38%)	12 (11.4%)	7 (3.76%)
Coryneform	49 (20.08%)	0	0
Gram-negative non lactose fermenters	16 (6.56%)	0	0
Non identified	3 (1.23%)	52 (?54.74)	77 (42.31)

Similarly the present study estimated about 32 (13.11%) Micrococcaceae, 18 (7.38%) Enterobacteriaceae, 49 (20.08%) Coryneform and 16 (6.56%) Gram-negative non-fermenters to occur in milk from infected cows.

Isolates from Udder Skin

Pseudomonas aeruginosa (10 = 9.5%) recorded the higher frequency of the isolated mastitis pathogens. One isolate could be identified for each of *S. aureus*, *Rhodococcus equi* and *Alcaligenes* (0.95%) and three isolates were *Proteus* sp. (2.85%). Although, *Bacillus* sp. showed the maximum rate of isolation from udder skin of the examined cows (15 = 14.25%), however only one isolate was identified as *B. cereus*. Moreover, the present study revealed high incidence of Enterobacteriaceae (12 = 11.4%) as shown in Table 1.

Isolates from Air of the Studied Farms

Five (2.7%) isolates of *S. aureus* were identified as pathogens from the air sedimented samples of the fences. The optimum number of the isolated organisms from air was *Bacillus* sp. (51 = 27.42%) followed by Micrococcaceae (27 = 14.52%). Other isolates include *Streptococcus* sp. (2 = 1.08%) and Enterobacteriaceae (7 = 3.76%) as shown in Table 1.

Antibiotics Susceptibility Tests

Table 2 shows that *Staphylococcus aureus* has high resistant towards tetracycline (27 = 79.41%) compared to that towards penicillin (10 = 29.41%) and amoxacillin- cluvenic acid (7 = 20.59%), while susceptible to other tested antibiotics. *Escherichia coli* and *K. pneumoniae* isolated during the present study were 100% resistant to penicillin, oxacillin and lincomycin. Moreover, *E. coli* showed relatively high resistant towards erythromycin (13 = 76.47%), medium resistance to tetracycline (5 = 29.41%), streptomycin and sulfamexazol-trimethoprim (3 = 17.65%) and lower rate of resistant towards kanamycin

Table 2: Antimicrobial resistant patterns of bacteria isolated from mastitis- infected cows

Antibiotics (%)	<i>S. aureus</i>	<i>Enterococcus faecium</i>	<i>E. coli</i>	<i>K. pneumoniae</i>
Enrofloxacin	0	6 (100)	0	0
Naladixic acid	35 (97.2)	6(100)	0	0
Oxacillin	0	6(100)	17(100)	23(100)
Erythromycin	0	0	13 (76.47)	23(100)
Streptomycin	0	0	3 (17.65)	0
Kanamycin	0	6(100)	1 (5.88)	0
Gentamycin	0	5 (83.33)	0	0
Lincomycin	0	6(100)	17(100)	23(100)
Penicillin	10 (29.4)	0	17(100)	23(100)
Amoxacillin-cluvenic acids%	7 (20.59)	0	0	0
sulfamexozal-trimethoprium%	0	1(16.66)	3 (17.65)	0
Tetracycline%	27 (79.41)	0	5 (29.41)	0
deoxycycline%	27(79.41)	0	0	intermediate sensitivity

Table 3a: Mastitis incidence among infected cow's quarters in dairy farms at Khartoum State

Area	Clinical (watery)	Subclinical	Purulent	Haemorrhagic	Dry quarters	Total
Omdurman	33	41	1	2	2	79
Khartoum	11	95	1	-	8	115
Khartoum North	26	47	2	2	3	80
Total	70	183	4	4	12	274

Table 3b: Numbers of infected quarters in different dairy farms at Khartoum State

Area	Right-fore	Left-fore	Right-hind	Left-hind	Total
Omdurman	22	19	18	20	79
Khartoum	18	19	22	21	115
Khartoum North	27	31	27	30	80
Total	67	69	67	71	274

(1 = 5.88%), while *K. pneumoniae* resisted erythromycin (100%). *Enterococcus faecium* showed 100% resistance for enrofloxacin, nalidixic acid, oxacillin, kanamycin and lincomycin. Five isolates (83.33%) resisted gentamicin, while only one isolate (16.66%) resisted sulfamexazol+ trimethoprim.

Incidence of Mastitis

The distribution of the disease among the quarters was more or less similar (Table 3a). However, the incidences of subclinical mastitis were found to be higher compared to the clinical form of the disease (Table 3b). The higher incidences of subclinical mastitis were found to be in Khartoum (Friesian), which showed the lowest incidences of the clinical mastitis. Similarly, the higher numbers of the dry quarters, which was indication of previous infection and that the Friesian cows are of high risk to mastitis incidences than the local and cross dairy breeds.

Discussion

The higher rates of subclinical mastitis incidence, obtained during the present study (Table 3) were similar to the previous study carried by Mohamed *et al.* (1993). However, a considerable variation in the incidence of clinical mastitis among the studied farms was found. This could be due, as stated by Sargeant (1998) to the majority of the first cases of clinical mastitis occur early in lactation and the risk of clinical mastitis increases with increasing parity. They concluded that environmental contagious and minor pathogens were all associated with cases of clinical mastitis. The higher incidences of total and subclinical mastitis was found in Khartoum as shown in Table 3b.

The incidence of the infection distributed more or less evenly between the quarters (Table 3a). However, Fitzgerald *et al.* (1997) reported that intramammary infection and high somatic cell count (SCC) were found less often in front and left quarters than in rear and right quarters. It was indicated that clinical mastitis associated with dry period infections was more likely to occur earlier in lactation than clinical mastitis infections. There was evidence of quarter susceptibility to IMI or the possibility that infection with one organism led to clinical mastitis with another (Green *et al.*, 2002).

The relatively high frequency of isolation of *S. aureus* during the present study was in accord with Kamata *et al.* (1990), Sargeant *et al.* (1998), Bezek (1998) and Sobiraj *et al.* (1997), as they have incriminated *S. aureus* as one of the most frequent causes of subclinical mastitis. Moreover, Fitzgerald *et al.* (1997) concluded that few specialized clones of *S. aureus* are responsible for the majority of the cases of bovine mastitis and that these colonies have abroad geographic distribution. However, staphylococcal poisoning, resulting from consumption of mastitis milk or milk extraneously contaminated, was frequently reported to occur in Sudan (Mustafa and Idris, 1976). The isolation frequency during this study was unlike that done by Rajala-Schultz *et al.* (2004). Since they reported that intra mammary infection in the Krauss Dairy Research Herd at the Ohio Agricultural Research and Development Center were mostly caused by the coagulase-negative staphylococci (78%).

Our present data revealed a low frequency of the isolation of *Arcanobacterium pyogenes* and *Streptococcus agalactiae* (0.41 and 0.82%), respectively, which were in accord with Sobiraj *et al.* (1997) and Sargeant *et al.* (1998) who isolated low frequency of *A. pyogenes*. However, this is in contrast to the findings of Kamata *et al.* (1990) and Bezek (1998). Moreover, Myllys *et al.* (1994) reported that the incidence of *Streptococcus agalactiae* has decreased, while, the incidence of Staphylococci, has increased. This could be due to, as they mentioned that external pressure, like changes in animal husbandry, including antimicrobial treatments and introduction of modern milking machines, act as a selective forces on the bacterial species that causes bovine mastitis.

The higher frequency of the rate of isolating Enterobacteriaceae, *Bacillus* sp. and coliforms were also supported Kamata *et al.* (1990), Bartlett *et al.* (1992) and Sobiraj *et al.* (1997). Moreover, the isolated Micrococcaceae may inhibit the infection by major pathogens (Table 1). This might be due to the protective effect of Micrococcaceae against other major pathogens (Lam *et al.*, 1997).

Pseudomonas aeruginosa (9.5%), *Proteus* sp. (2.8%), *Bacillus* (14.25%) and Enterobacteriaceae (11.4%) isolated from udder skin of the cows, as shown in Table 1 might be due to the presence of the mud all over the body of the cows. Since most of the samples were collected during fall season. Similarly Murphy and Boor (2000) reviewed that teats and udders of cows inevitably become soiled when animals are held in muddy barnyards or when cows are lying in stalls. However, of the pathogenic bacteria, only one strain of *S. aureus* (0.95%) was identified. Similarly, the samples from air revealed only 3% *S. aureus* of the isolated strains. Further, Roberson *et al.* (1998) reported that the major source of *S. aureus* intramammary infection in heifers at parturition are milk and heifer body sites. They also reported that *S. aureus* from the environment was never the sole possible source for *S. aureus* from heifer colostrums. Moreover, Murphy and Boor (2000) stated that organisms commonly associated with environmental mastitis (e.g., *St. uberis* and *St. dysagalactiae*) are found in the cow's environment and thus also may influence bulk milk bacterial counts through means other than mastitis infections.

Tetracycline showed the highest resistance percent to the tested isolates (Table 2). This was in accord to Mehranz (1998) and Perreten *et al.* (1998). However, it was in contrast to Vasil (1994), who demonstrated only 4.1-3.9% of *S. aureus* resistant to tetracycline. Further, similar, to the present

findings, they reported 33-20.3% penicillin resistant (Table 2.). Moreover, Adesiyun, (1995) reported 59 (23.6%) of *S. aureus* were found to be resistant to penicillin. The penicillin resistant could be attributed to milk fat globules (Ali-Vehmas *et al.*, 1997) or because 40% of those bacteria are penicillinase-positive (Sobiraj *et al.*, 1997). Hence In high SCC cows, it is important to select cows appropriate for treatment (as opposed to culling), and there is a good clinical justification for the use of antibiotics to treat existing IMIs. In low SCC cows, teat sealants provide a viable alternative to antibiotic DCT, and careful consideration should be given to their use (Bradley and Green 2004). However, *Staphylococcus aureus* isolated during the present study, were found to be sensitive to minocycline.

The Enterococci, isolated from mastitis cases showed a high resistant towards antibiotics tested (Table 2). This was similar to Lutfor *et al.*, (1998) and Hryniewicz *et al.* (1998).

Most of the isolates tested, when conducting this study (Table 2) revealed high percentages of susceptibility to antibiotics. It is concluded that, in Sudan (Khartoum State) most of mastitis causing bacteria were highly susceptible to antimicrobial agents (with the exception of tetracycline and penicillin). This might be due to unavailability of some drugs. Moreover, the use of antibiotic therapy is not common among most of the dairy farms. Further, we could also concluded that although pathogenic *S. aureus* were isolated from the air of the farms and teat skin of the cows it was at non-significant rate and in most cases, were not the causative agents of the disease in the examined herds (data not shown). Similarly, their presence is of low frequency (3 and 0.95%, respectively). However this might still it may create antibiotics resistant hazards to animals and human as stated by Manie *et al.* (1999) that resistant bacteria can be transferred to humans through the consumption of rare and raw beef and non pasteurized milk, thus rendering the resultant food-related infections difficult to treat.

The present study recommended that contamination at the site of production must be avoided because of its deleterious effects on the keeping quality of the milk and its suitability for further processing into products. Further epidemiological studies on the occurrence of mastitis and its associated predisposing and environmental factors are needed to adopt the suitable control measures. Antibiotic resistant is urgently need to be assessed in order to evaluate and minimize its health risk hazards.

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