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Incidence of Some Potential Pathogens in Raw Milk in Khartoum North (Sudan) and Their Susceptibility to Antimicrobial Agents

Sanaa O. Yagoub, Nazik E. Awadalla and Ibtisam [1]E. M. El Zubeir
Department of Microbiology and Molecular Biology, Faculty of Science and Technology, El Neelain University,Khartoum, Sudan
[1]Department of Dairy Production, Faculty of Animal Production, University of Khartoum, Khartoum North, Sudan

Abstract: The most predominant bacterium in raw milk samples investigated during the present study was *Staphylococcus aureus,* 21 isolates (30%) . Moreover, Gram- negative bacteria identified as *Citrobacter spp.*, 15 (21.43%) , *Shigella spp.*, 14 (20%), *E. coli*, 10 isolates (14.28/%), *Enterobacter spp.*,9 (12.86%), and *Salmonella spp.*, 1, (1.43%) . *Staphylococcus aureus* count in the milk collected from the factory was (6×10^3 –1.2×10^6). *Salmonella spp.* count was 5×10^4 in the milk collected from the factory. *Shigella spp.*, *Enterobacter spp.* and *Citrobacter spp.* were predominant in the milk collected from the factory (7×10^5 –1.4×10^6). On the other hand, the *E. coli* count was higher in the milk collected from the collection points (7×10^4 -7.5×10^6). In the present study, most of the bacteria isolated from the raw milk showed a wide range of multiple resistance to the tested antimicrobial agents. Penicillin, clindamycin, amoxicillin and ampicillin showed the highest resistance. Chloramphenicol showed the highest antimicrobial activity against the test organisms followed by gentamicin , novobiocin and carpencillin.

Key words: Potential, Pathogens, Khartoum, Susceptibility, Antimicrobial

Introduction

Fresh milk obtained from healthy udder under sanitary conditions contains relatively few microorganisms but subsequently becomes contaminated by man, his practices and the environment (International Dairy Federation, 1994). Unpasteurized milk contains a wide range of bacteria, principally those causing mastitis, and also the faecal flora arising from udder contamination (Ministry of Agriculture, Fisheries and Food, 1998). Selim and Cullor (1997) found that *Streptococci* and coliforms are the most dominant isolated bacteria from milk followed by *Staphylococcus spp.* Moreover, they reported that *E. coli* was the most common isolated Gram- negative bacteria. *Escherichia coli* in many cases, may not only contaminate food, but grows in it and reaches high numbers, particularly in tropical countries or in the absence of refrigeration (Wood *et al.*, 1998). The Gram- negative organisms associated with lowering of milk quality included *Citrobacter spp.*, *Enterobacter spp.*, *E. coli* and *Klebsiella spp.* (Jayarao and Wang, 1999).

Claims that the practices of dosing animals with subtherapeutic concetrations of antibiotics that promote the development of antimicrobial resistance have been recently increased (Amabile-Cuevas *et al.*, 1995). The infection of man with bacteria resistant to antimicrobials is a cause of increasing concern. Some of these antimicrobial- resistant bacteria reach man in food and particularly in food of animal origin to cause diseases directly or act as potential sources of antimicrobial resistance for human pathogens (Ministry of Agriculture, Fisheries and Food, 1998).

The main carriers of antimicrobial resistance in milk are the *Staphylococci*, some of this resistance is transferable (Muhammad *et al*, 1993). Schuman *et al.* (1989) described *Salmonella typhimurium* strain with an unusual plasmid profile isolated from pasteurized milk and from asymptomatic patient during 1985. This *S. typhimurium* strain developed multiple antibiotic resistance (to 14 agents). Johnston *et al.* (1983) reported that antibiotic resistant *E. coli* was found in 10.6% of milk samples, most of which resisted more than one antibiotic. Few Shigellae can be found in animals and food- borne *Shigella* infections are likely to be of human origin (Hoge *et al.*, 1996).

The present study aimed at estimating the numbers of some potential pathogens and determining antibiotic resistance spectra of those microbial flora of raw milk.

Materials and Methods

Source and collection of milk samples: A total of 90 bulk milk samples were collected from the Butana Dairy Factory, farms and milk collection points and located in Hillat Kuku, Khartoum North. The samples were collected in sterile containers after thorough mixing of the milk under aseptic conditions, immediately kept in an ice box and transported to the Laboratory, Department of Microbiology and Molecular Biology, School of Biotechnology, El Neelain University, Khartoum.

Isolation and identification: The milk samples were serially diluted according to the method of Cheesbrough (1984). One ml of the selected dilutions was transferred to duplicate plates of the selected media using sterile pipettes. Media prepared according to the manufacturer's instructions were used for enumeration of isolated bacteria. Mannitol salt agar was used for *S. aureus*, MacConkey's agar was used for coliform bacteria, Eosin methylene blue was used for *E. coli* and and Salmonella- Shigella agar and Tellunite citrate agar (Oxoid) were used for *Salmonella spp.* and *Shigella spp.*. The plates were incubated at 37? C for 24 hours. Purified isolates were identified according to their morphological, cultural, biochemical and sugar fermentation characteristics (Barrow and Felthman, 1993).

Antibiotic sensitivity test: The purified isolates were subjected to an *in vitro* antimicrobial susceptibility test using the diffusion method described by Cheesbrough (1984). Penicillin G (10 IU), ampicillin (10 μg/disc), erthromycin (15μg/disc), chloramphenicol (30μg/disc), amoxicillin (25μg/disc), carpencillin (100 μg/disc), novobiocin (30μg/disc), clindamycin (2μg/disc) and gentamicin (10μg/disc) were the selected antibiotics.

Results and Discussions

Some potential pathogenic bacteria were isolated from raw milk samples produced and marketed in Kuku area. The isolates were as follows: *Staphylococcus aureus,* 21 (30%), *Citrobacter spp.*, 15 (21.43%), *Shigella spp.*, 14 (20%), *E. coli,* 10 (14.28%), and *Enterobacter spp.*, 9 (12.86%). However, one isolate (1.43%) was found in milk and identified as *Salmonella spp.* (Table 1).

Table 2 shows that the count of *Staphylococcus aureus* was found in the range of $5\times 10^3 - 1.2\times 10^6$, 6×10^3- 12×10^5 and 2×10^3-1×10^5 from factory, collection points and farms, respectively. *Salmonella spp.* count revealed 5×10^4, while the *Shigella spp.* was estimated at 1.1×10^6, 1×10^5, 1×10^5, respectively. The ranges of *Shigella spp.* were found to be 7×10^5- 1.4×10^6, 2×10^3 - 1×10^5 and 1×10^5 - 3×10^5. *Escherchia coli* mean count was recorded as 1×10^6, 7.3×10^6 and 2×10^5, while the ranges of *E. coli* were found to be $1.1\times 10^4 - 2.9\times 10^6$, $7\times 10^4 - 7.5\times 10^6$ and 1×10^3 - 9×10^5. *Enterobacer spp.* mean counts were reported as 5×10^6, 1×10^5 and 3×10^5 and the ranges were found to be 5×10^4 - 2×10^6, 1×10^5 and $3\times 10^4 - 1.5\times 10^6$. The *Citrobacter spp.* revealed mean counts of 6×10^5, 5×10^4 and 4×10^5 and the ranges were found to be 5×10^4- 2×10^6, 2×10^4- 2×10^6 and 2×10^5, respectively.

Staphylococcus aureus was found to be either sensitive (76.2%) or intermediately sensitive (23.8%) to chloramphenicol, novobiocin revealed 61.9% sensitivity and 28.6% intermediate sensitivity. Gentamicin revealed 47.6% sensitivity and 42.9% intermediate sensitivity for the tested *S. aureus* isolates. Similarly, carpencillin revealed 42.9 and 28.6% sensitivity and intermediate sensitivity, respectively (Table 3). However, penicillin, ampicillin, amoxicillin and clindamycin showed 66.7%, 57.1%, 57.1% and 47.6% resistance by the tested *S. aureus* isolates. In the present study, isolated *Salmonella spp.* showed resistance to ampicillin, amoxicillin and clindamycin. *Shigella spp.* showed the best sensitivity towards chloramphenicol (92.9% and 7.1%) followed by gentamicin at 64.3% and 35.7% and carpencillin at 78.7% and 14.3%, respectively, for sensitivity and intermediate sensitivity. However, ampicillin, amoxicillin and clindamycin showed resistance at 92.9%, 92.9% and 85.7%, respectively. *Escherichia coli* isolated during the present study showed similar trends, since 90% and 80%, 70% and 20%, 60% and 30% and 50% and 40% sensitivity and intermediate sensitivity towards carpencillin, gentamicin, chloramphenicol and novobiocin, respectively. On the other hand, clindamycin showed 80%, ampicillin 60%, penicillin 50% and erythromycin 50% resistance towards *E. coli.*

Citrobacter spp. also showed best results with chloramphenicol (60% and 40%) and gentamicin (53.3% and 46.7%) followed by carpencillin (60%, 26.7%). Moreover, those organisms showed resistance to penicillin (80%), novobicin (60%), amoxicillin (53.3%) and clindamycin (46.7%). Similarly, *Enterobacter spp.* were more susceptible to chloramphenicol (77.77% and 33.33%) and carpencillin (66.67%, 33.33%) then to gentamicin, erthromycin and novobiocin (44.44% and 44.44%). On the other hand, *Enterobacter spp.* showed high resistance to penicillin (66.67%) and ampicillin (55.56%).

The present study has shown the isolation of six potential and opportunistic pathogens from the three different sources of collected raw milk samples. Their presence in milk suggested contamination from various sources, which may include animal, human, environment, utensils and others (Adesiyun *et al.*, 1997; Giovannini, 1998 and Murphy and Boor, 2000). Several animal pathogens can cause human disease and they are well known to be transmitted to human by consumption of raw milk (International Dairy Federation, 1994).The presence of *Staphylococcus aureus* and *E. coli* supported the findings of Selim and Cullor (1997), who also reported that unpasteurized milk should be used with caution, because it may contain a high number of bacteria that may be pathogenic to cattle and human.

The high numbers of the isolated microorganisms in milk during the present study indicated that those microorganisms not only contaminate milk but also multiply and grow on it. This might be due to the fact that the milk is a good nutritive medium for the growth of the microorganisms, especially with poor sanitary procedures (Adesiyun *et al.*, 1997) and the lack of the cooling facilities (Murphy and Boor, 2000). Moreover, the tropical hot climate aggravates the conditions (Wood *et al.*, 1998) . The members of coliforms bacteria constituted 70% of the isolates; *E. coli, Citrobacter spp., Enterobacter spp.* accounted for 14.28%, 21.43% and 12.86% of the isolates, respectively. Those numbers were higher than those reported by Jayarao and Wang (1999). The presence of coliforms group in raw milk is generally regarded as faecal pollution (Adesiyun *et al.*, 1997) .

The higher counts of Gram- negative bacteria (*E. coli, Citrobacter spp., Enterobacter spp. and Shigella spp.*, were more or less similar to the findings of Jayaro and Wang (1999). Moreover, Thomas and Thomas (1973) considered counts greater than 10^5 cfu/ml of raw milk as poor quality.

Table 1: Number and percentage of the isolated bacteria from raw milk samples

Bacterial species	Number of isolates	Percetage of islates
S. aureus	21	30.00
Citrobacter spp.	15	21.43
Shigella spp.	14	20.00
E. coli	10	14.28
Enterobacter spp.	9	12.86
Salmonella spp.	1	1.43
Total	70	100.00

Table 2: Comparison of bacterial load of raw milk samples collected from different sources

	Factory		Collection Point		Farms	
	Mean	Range	Mean	Range	Mean	Range
S. aureus	4×10^5	5×10^3- 1.2×10^6	1×10^5	6×10^3- 12×10^5	7×10^5	2×10^3- 1×10^5
Salmonella spp.	5×10^4	5×10^4	0	0	0	0
Shigella spp.	1.1×10^6	7×10^5- 1.4×10^6	1×10^5	2×10^3- 1×10^5	1×10^5	1×10^5-10^5*E.*
coli	1×10^6	1×10^4- 2.9×10^6	7.3×10^6	7×10^4- 7.5×10^6	2×10^5	3×10^3- 9×10^5
Enterobacter spp.	5×10^6	5×10^4- 2×10^6	1×10^5	1×10^5	3×10^5	5×10^4-$.5\times10^6$
Citrobacter spp.	6×10^6	5×10^4- 2×10^6	5×10^4	2×10^4- 2×10^6	4×10^5	4×10^5

Table 3: Antimicrobial susceptibility tests for the isolated potential pathogenic bacteria from raw milk samples

	S. aureus			Salmonella spp.			Shigella spp.			E. coli			Citobacter spp.			Enterobacter spp.		
	Percentage			Percentage			Percentage			Percentage			Percentage			Percentage		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Gentamicin (GN 10)	47.6	42.9	9.5	100	0	0	64.3	35.7	0	70	20	10	53.3	46.7	0	44.4	44.44	11.12
Carpencillin (Py 100)	47.9	28.6	28.6	100	0	0	78.6	14.3	7.1	80	20	0	60	26.7	13.3	66.67	33.33	0
Erthromycin (E15)	23.8	38.1	38.1	0	100	0	0	71.4	28.6	0	50	50	33.3	40	26.7	44.44	44.44	11.12
Ampicillin (AM 10)	14.3	28.6	57.1	0	0	100	0	7.1	92.9	20	20	60	13.3	53.3	33.3	0	44.44	55.56
Amoxicillin (AX 25)	28.6	14.3	57.1	0	0	100	0	7.1	92.9	50	10	40	33.4	13.2	53.4	33.33	44.44	22.23
Clindamycin (DA 2)	28.6	28.6	47.6	0	0	100	7.1	7.1	85.8	0	20	80	20	33.3	47.7	22.22	55.56	22.22
Novobiocin (NV 30)	61.6	28.6	9.5	100	0	0	7.1	92.9	0	50	40	10	13.3	26.7	60	44.44	44.44	11.12
Peniclin (P 10)	19.1	14.3	66.7	100	0	0	7.1	50	42.9	20	30	50	6.7	13.3	80	0	33.33	66.67
Chloramphenicol (C 30)	76.6	23.8	0	100	0	0	92.1	7.1	0	60	30	10	60	40	0	77.77	22.22	0

S = Sensitive I = Intemediate R = Resistance

The incidence of isolated bacteria was found to be higher in the factory compared with farms and collection points. This may indicate extra sources of contamination like milk utensils, equipment and containers of transportation (Murphy and Boor, 2000). Moreover, *Enterobacter spp.* and *Citrobacter spp.* were found in higher counts in the factory samples. On the other hand, the higher count of *E. coli* followed by *Citrobacter spp.* in the collection points might refer to improper public health measurement and sanitary and poor cleaning of people concerned with milk marketing in addition to the primitive system of transportation and marketing practiced in Sudan. The number of coliform bacteria was found to exceed the legal standard which ranges from 50-750 cfu/ml as reported by Murphy and Boor (2000). *Staphylococcus aureus* may originate from mastitic animals (Mohamed *et. al.*, 1997) or human sources (Chambers, 1997). Moreover, *S. aureus* was found to be involved in food poisoning (Adesiyun *et al.*, 1997).

Most of the bacteria isolated from the raw milk in the present study, showed a wide range of multiple resistance to the tested antimicrobial agents. Penicillin, clindamycin, amoxicillin and ampicillin showed the highest resistantce.

Use, misuse and often abuse of antimicrobial agents have encouraged the evolution of the bacteria towards resistance resulting in therapeutic failure (Straut, *et. al.*, 1995). Furthermore, resistance can be transferred between different species of bacteria mostly in related genera e.g Enterobacteriaceae (Ministry of Agriculture, Fisheries and Food, 1998).

The resistance of *Salmonella spp.* to many antimicrobial agents supported the findings of Schuman *et al.* (1989).

The resistance of *Staphylococcus aureus, Shigella spp., Citrobacter spp.* and *Enterobacter spp.* to ampicillin, amoxicillin and clindamycin is alarming since those antibiotics are commonly used and available. Although *Staphylococcus aureus* showed high resistance to B- Lactamase (penicillin, ampicillin, and amoxicillin) in the present study, novobiocin showed low resistance. This might be due to the uncommon use of this antibiotic in the local farms. It is suggested that isolated *Staphylococcus aureus* might belong to different serotypes or strains. Singh and Boxi (1982) reported that chloramphenicol was the most effective and pencillin was the least effective antimicrobial agents. *Escherichia coli* due to their production of B- Lactamase enzymes (Brooks *et al.*, 1991), showed relative resistance to penicillin.

We conclude that chloramphenicol showed the best antimicrobial effect aganist the tested organisms followed by gentamicin, novobiocin and carpencillin. This might be due to unavailability of those drugs in most of the cases or their high price. *Citrobacter spp.* showed relatively higher resistance to novobiocin.

The high incidence and counts of potential pathogens were accompanied by multiple resistance to some tested antimicrobial agents. This might create health hazards for consumers and handlers of such milk particularly because the transfer of the resistance via food chain and gene encoding resistance were very well documented. Hence educational and extension programs for consumers, producers and users about the risks of consumption of raw milk are needed in order to evaluate and correct this situation.

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