World Journal of Dairy & Food Sciences 4 (1): 65-69, 2009
ISSN 1817-308X

Antimicrobial Resistance of Bacteria Associated with Raw Milk Contaminated by Chemical Preservatives

Ibtisam E.M El Zubeir and O.A.O. El Owni

Department of Dairy Production, Faculty of Animal Production, University of Khartoum, Khartoum North, P.O. Box 32 Postal Code 13314, Sudan

Abstract: This study was carried out on two hundred and forty raw milk samples collected randomly from farms and sales points in Khartoum State. One-hundred and twenty milk samples were collected during each summer and winter from the same sources. The milk samples were examined for the presence of some chemical preservative and isolation and identification of microorganisms from antibiotic contaminated milk. Moreover susceptibility of isolated bacteria towards some antibiotics was estimated. The chemical preservative residues examination included the detection of formaldehyde, hydrogen peroxide, boric acid, alkalinity of ash and detection of antibiotics and sulphonamide residue. In this study it was found that 5 (2.08 %) of the milk samples contained formaldehyde, moreover the result showed that the milk samples from sale points showed higher values compared to that from the farms. One sample (0.41%) was positive to the presence of hydrogen peroxide and 4 (1.6%) showed alkalinity of ash. Both were detected only in the milk samples collected from the farms. However all the milk samples examined contained no boric acid. On the other hand 30 (12.25 %) and 16 (6.66%) milk samples were contaminated with antibiotic and sulphonamide respectively, with the highest incidences in the milk samples collected from Khartoum compared to that collected from Omdurman and Khartoum North. The high incidence of antibiotic residues in the milk samples collected from venders might suggest the addition of antibiotics to the milk in order to prolong its shelf life. The isolated bacteria were identified as *Staphylococcus aureus (*8; 26.66%), *Streptococcus pyogenes* (4; 13.33%), *Corynebacterium ovis* (2; 6.66%), *E. coli* (5; 16.66%), *Citrobacter Kasseri* (6; 20%), *Klebsiella aergenes* (*2; 6.66*%), *Pseudomonas* aeruginosa (2; 6.66%) and *Proteus mirabilis* (3.33%). The isolated bacteria showed wide range of multiple resistances. The highest resistance was towards penicillin (63.33%), collxacillin (63.33%) clindamycin (56.66%) and ampicillin (56.66%), while Chloramphenicol (3.33%) showed the highest antimicrobial activity against the test organisms followed by pipercillin (13.33%) and gentamicin (20.00%).

Kew words: Raw milk · Farms · Sales points · Chemical preservative · Bacterial species · Antibiotics resistance

INTRODUCTION

Preservatives are used to preserve food by preventing growth of microorganism and subsequent spoilage including fungus, mould and rope inhibitors [1]. Chemical preservation works either as direct microbial poisons or by reducing pH to a level of acidity that prevents the growth of microorganism [2]. The hydrogen peroxide treatment was found to be an effective and affordable means by which farms in tropical developing countries could extend the keeping quality of milk during transportation to the market or processing plants [3]. Moreover 0.04 to 0.05% of hydrogen peroxide is enough to preserve milk up to 24 hours [4].

Addition of some antibiotics into feed additive as growth promoters may enhance a large pool of resistant organisms and resistant genes, which may contribute to the development and transmission of antibiotic resistant bacteria [5-7]. However direct transfer of antibiotics resistant microorganisms to humans through consumption of milk is unlikely because most milk is pasteurized [8]. The relationship between antibiotic residues in milk and the development or transfer of resistant pathogens appears to be hypothetical [9].

Corresponding Author: A.E.A. Saeed, Department of Dairy Production, Faculty of Animal Production, University of Khartoum, Khartoum North, P.O. Box 32 Postal Code 13314, Sudan

Milk can be contaminated with feed pathogens that exhibit resistance to antibiotic and raw milk products have been implicated as mechanisms for transferring antibiotic resistant organism from farm environments to humans [10].

Safe milk should not contain residues of antibiotic [11]. These residues are a result of treating dairy cattle with antibiotic and not with holding milk [12, 13]. Antibiotic residues may also impact the manufacturing process of milk products [14, 15]. Antibiotic residues occur in milk supplies throughout the world, in some relatively unregulated markets, antibiotic residues may exist in 8-15% of total bulk tank loads [16].

Purpose of antibiotic sensitivity testing is to determine the susceptibility of bacteria to various antibiotics. This standardized test is used to measure the effectiveness of a variety of antibiotics on a specific organism in order to prescribe the most suitable antibiotic therapy [17].

The present study was initiated with the objectives of estimating preservative in milk supply to Khartoum State and detection of antibiotic residue in milk. Isolation and identification of some pathogenic bacteria associated with antibiotic contaminated milk were also investigated.

MATERIALS AND METHODS

Sources and Collection of Milk Samples: This investigation was based on collection of 120 raw milk samples during winter season; January – February 2005 (120 samples) and 120 raw milk samples during summer season. June – July 2005 from farms (n= 9) and sale points (n= 9) in Khartoum State. The milk samples were collected from Khartoum (80 samples), Khartoum North (80 samples) and Omdurman (80 samples) during summer and winter (forty samples each). The milk samples were collected into clean sterile bottles and transported in an ice box to the laboratory of the Department of Dairy Production, Faculty of Animal Production, University of Khartoum, where examinations of the milk samples were done.

Detection of Chemical Residues: The presence of formaldehyde was indicated by the appearance of the blue color on the addition of a 1% ferric chloride solution in concentrated sulphuric acid and the presence of hydrogen peroxide was indicated by the addition of a small amount of Paraphenylene diamine solution to the milk [18]. The presence of boric acid involves the reaction between it and Polyhydroxy compounds such as glycerol to from an organic boric acid and the alkalinity of ash was done according to Foley *et al*. [18].

Detection of Antibiotic Residues: Petri dishes continuing nutrient agar inoculated with *Bacillus subtitles* were used to estimate antibiotic residues in milk samples [19]. Total antibiotic and sulphonamide kits (Bio-x diagnostic, Belgium) were also used for the detection of antimicrobial substance in milk samples according to the manufactures instructions.

Microbiological Examination: Sterilization, preparation of media and culturing methods were done according to the standard procedures [20]. The predominated microorganisms from morphologically different colony types; from milk contaminated by antibiotic residues were selected from plate count agar. The identification of pure bacterial isolates was done as described before [20, 21].

Sensitivity Test: Sensitivity test was done on diagnostic sensitivity test media using Mueller and Hinton agar media [22]. The results were recorded as resistant, intermediately sensitive or sensitive according to the diameter length as described by manufacturers.

RESULT AND DISCUSSION

Milk samples contaminated by formaldehyde were found to be high in the sale points compared to those collected from farms (Table 1). This could be due to the milk sellers who might add chemical preservatives to milk in order to increase its shelf life. However most of the samples which showed alkalinity of ash and hydrogen peroxide were found in the milk samples which were collected from the farms (Table 1). A decrease in total bacterial counts at six hours after the addition of hydrogen peroxide was reported [23]. It was also observed that the keeping quality of milk with hydrogen peroxide increased compared with untreated milk [4]. The boric acid was not detected in the milk samples collected during the present study.

Table 1 showed that positive milk samples for antibiotic and sulphonamide residues were high compared to those collected from many farms, which might be due to misuse of antibiotics [11]. They noticed that in most of the farms, the farmers are used to mix milk from many cows in bulk tank or container, hence the concentration of many antibiotics in samples of milk were diluted. Similarly the sellers in the sale points also mix all milk collected from many farms. In spite of this numbers

Table 1: Incidence and frequencies of chemical preservation in milk samples

Location	Sources	Formal dehyde	Hydrogen peroxide	Boric acid	Alkalinity of ash
Khartoum	Farms	0	1 (2.5%)	0	0
	Sale points	2 (5.0%)	0	0	0
Total		2 (2.5%)	1(1.25%)	0	0
Khartoum North	Farms	1 (2.5%)	0	0	3 (7.5%)
	Sale points	2 (5.0%)	0	0	0
Total		3 (3.7%)	0	0	3 (7.5%)
Oumderman	Farms	0	0	0	0
	Sale points	0	0	0	1(2.5%)
Total		0	0	0	1 (1.25%)
Khartoum State*		5 (2.08%)	1 (0.41%)	0	4 (1.6%)

*Over all mean (summer and winter) for all samples

Table 2: Incidence and frequencies of antibiotic and sulfonamide in milk samples collected from different locations in Khartoum State

Location	Sources	Antibiotic kits	Disk assay	Sulphanomide
Khartoum	Farms	8 (20%)	8 (20%)	2 (5.0%)
	Sale points	5 (12.5%)	4 (10%)	3 (7.5%)
Khartoum State		13 (16.25%)	12 (15%)	6 (7.5%)
Khartoum North	Farms	3 (7.5%)	3 (7.5%)	2 (5.0%)
	Sale points	5 (12.5%)	6 (15%)	3 (7.5%)
Khartoum State		8 (10%)	9 (11.25%)	5 (6.25%)
Oumderman	Farms	4 (10%)	2 (5.0%)	3 (7.5%)
	Sale points	5 (12.5%)	5 (12.5%)	2 (5.0%)
Khartoum State		9 (11.25%)	7 (8.75%)	5 (6.25%)
Khartoum State*		30 (12.5%)	28 (11.66%)	16 (6.66%)

*Over all mean (summer and winter) for all samples

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

Bacteria	*Staphylococus aurous* (%)			*Streptococcus pyogenes* (%)			*Corynobacterium ovis* (%)			*Escherichia coli* (%)			*Citrobacter koseri* (%)			*Klebsiella aerogenes* (%)			*Pseudomonas aeruginosa* (%)			*Proteus Mirabilis* (%)		
Antibiotic	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Gentamicin (gm10)	62.5	12.5	25	25	25	50	50	50	0	60	20	20	66.7	33.3	0	50	50	0	0	0	100	100	0	0
Penicillin	37.5	12.5	50	25	0	75	50	0	50	20	20	60	16.7	33.3	50	50	0	50	0	50	50	0	0	100
Cephalexin (pr30)	25	62.5	12.5	25	50	25	0	100	0	20	40	40	16.7	50	33.3	50	50	0	50	0	50	0	100	0
Tetracycline (te30)	37.5	50	12.5	75	25	0	0	100	0	40	0	60	33.3	16.7	50	50	0	50	50	0	50	100	0	0
Colxacillin (cx5)	25	12.5	62.5	0	25	75	100	0	0	20	0	80	0	33.3	66.7	0	50	50	50	0	50	0	0	100
Erythromycin (e15)	25	37.5	37.5	25	25	50	50	0	50	0	50	50	16.6	16.6	66.7	0	50	50	50	50	0	100	0	0
Clindamicin (cd2)	12.5	25	62.5	0	25	75	0	50	50	0	40	60	16.6	50	33.3	50	0	50	0	50	50	0	100	0
Chloramphenicol (ch30)	75	25	0	75	25	0	100	0	0	40	40	20	66.7	33.3	0	50	50	0	0	0	100	100	0	0
Pipercillin (pc100)	75	12.5	12.5	50	25	25	50	50	0	60	40	0	66.7	33.3	0	50	0	50	0	50	50	0	100	0
Ampicillin (as20)	12.5	25	62.5	0	25	75	50	50	0	0	60	40	16.6	16.6	66.7	0	50	50	0	0	100	0	0	100

S: sensitive I: intermediate R: resistance

of positive samples of raw cow milk (Table 2), it was demonstrated that venders are used to add antibiotics to milk in order to preserve and increase shelf life, which supported the previous work [11]. The residues of antibiotics are harmful to humans, resulting into therapy failure and development of antibiotic resistant organisms [6, 24, 25]. Moreover antibiotics residues in milk are undesirable for public health and for technological reasons [14]. The presence of antibiotics in the milk samples of most of the studied farms might be due to the fact that during winter when weather becomes cold, diseases such as pneumonia increased and farmers used antibiotics to treat animals, therefore antibiotics residue transferred into milk. Also it might indicate the increase of awareness among the animal owners, which could be attributed to the increased education levels and increased veterinarian visits during animal treatment. The high awareness was also observed from our informal discussion with some of the farmers and milk sellers who supply clean milk.

The present study showed the isolation of 8 potential and opportunistic pathogens from milk samples obtained from both farms and sales points. The isolates were found as *Staphylococcus aureus (*8; 26.66%), *Streptococcus pyogenes* (4; 13.33%), *Corynebacterium ovis* (2; 6.66%), *E. coli* (5; 16.66%), *Citrobacter Kasseri* (6; 20%), *Klebsiella aerogenes* (2; 6.66%) and *Pseudomonas aeruginosa* (2; 6.66%). However one isolate (3.33%) found in milk was identified as *Proteus mirabilis* (Table 3). More or less similar bacteria from raw milk from both farms and sales points in Khartoum North were isolated previously [25]. The presence of those bacteria in milk suggested contamination from various sources, which may include animal, human, environment, utensils and other [7]. The high numbers of the isolated microorganisms not only contaminate the milk but also multiply and grow in it [26]. This might be due to the fact that milk is a good nutritive medium for the growth of microorganisms, especially with poor sanitary procedures [26, 27] and lack of the cooling facilities [7]. Moreover the higher incidence of isolated bacteria was found to be *Citrobacter kasseri* followed by *E. coli* (Table 3). This might be due to the improper hygiene and sanitation, poor cleaning and marketing environment in addition to primitive system of transportation and marketing [28]. The presence of *Corynobacterium ovis* might indicate that the milk from sheep was mixed with cow's milk as it was noticed that sheep and cows were found in same farm.

Multiple resistances of the isolated bacteria were found in the tested antimicrobial agents. Penicillin, cilndanycin and ampicillin showed the highest resistance (Table 3). This is an alarming result since those antibiotics are commonly used in most of farms in Sudan. *Staphylococcus aureus* showed high resistance to chloramphencol in the present study. The low resistance towards Chloramphenicol might be due to the uncommon use of this antibiotic in the local dairy farms, moreover penicillin was reported as the least effective antimicrobial agent [24, 25, 29]. Chloramphenicol showed the best antimicrobial effect against the tested organisms followed by gentamicin and piperacillin.

This study suggested that more efforts are needed to enhance and promote farms and sale points of milk by using screening confirmatory tests at sales points and farms. Moreover, the ministries concerned should adopt comprehensive strategy for ensuring a safe supply of good quality milk. These strategies should include promoting knowledge of farmers' standards through training and extension and the adoption of grading and quality testing of milk. Ultimately, the milk testing programs should become component of the quality process that should focus on production of high quality milk.

ACKNOWLEDGMENT

This study is part of a project supported by Ministry of Higher Education and Scientific Research, Sudan. We would like to acknowledge BIO- X Diagnostic Company (Belgium) for providing the antibiotics kits. Deepest thanks are extended to the owners, of the farms and sale points from whom, we collected milk samples. Thank is also extended to Dr. Abdel basit Elamin, University of Nyala for his assistance during statistical analysis of the data.

REFERENCES

1. Vollhardt, K.P.C. and N.E. Schore, 1998. Chemical preservative for additive. Thorson publisher limited. Third edition.
2. Australian Academy of Science, 2004. Chemical preservation. Cited in: http://www.yahoo.com/ search? publications/abstracts/september 2004.htm.
3. Odoi, A., 2003. Effectiveness and affordability of hydrogen peroxide in milk preservation under tropical conditions. Milchwisenschaft, 58 (1-2): 65-67.
4. Saha, B.K., M.X. Ali, M. Chakraorty, Z. Islams and A.K. Hira, 2003. Study on the preservation of raw milk with hydrogen peroxide for rural dairy farmer. Pakistan J. Nut., 2(1): 39-42.
5. Mitchell, M., B. Bodkin and J. Martin, 1995. Detection of beta lactams antibiotic in bulk tank milk. J. Food Protection, 58: 577-578.
6. Manie, T., V.S. Brozel, W.J. Veith and P.A. Gouws, 1999. Antimicrobial resistance of bacterial flora associated with bovine products in South Africa. J. Food Protection, 62: 615-618.
7. Murphy, S.C. and K.J. Boor, 2000. Trouble shooting sources and causes of high bacteria counts in raw milk. Dairy Food and Environmental Sanitation, 20(8): 606-611.
8. Teuber, M. and V. Perreten, 2000. Role of milk and meat products as vehicles for antibiotic resistant bacteria. Acta Vet. Scand. (Suppl.), 93: 75-87.
9. Tikofsky, L.L., J.W. Barlow, C. Santisteban and Y.H. Schukken, 2003. A comparison of antimicrobial susceptibility patterns for *Staphylococcus aureus* in organics and conventional dairy herds. Micro Drug Resistance Mech. Epi. and Disease, 9 (Suppl.): 539-545.

10. Kalman, M., E. Szollosi, B.M. Czermann Zimanyi and S. Szekeres, 2000. Milk borne campylobacter infection in Hungry. J. Food Protection, 63: 1426-1429.
11. Said Ahmad, A.M.M., Ibtisam, E.M. El Zubeir, O.A.O. El Owni and M.K.A. Ahmed, 2008. Assessment of microbial loads and antibiotic residues in milk supply in Khartoum State, Sudan. Res. J. Dairy Sci., 2(3): 36-41.
12. Sargean, J.M., Y.H. Schukken and K.E. Leslie, 1998. Ontario milk somatic cell count reduction progress and outlook. J. Dairy Sci., 81: 1545-1554.
13. Norman, H.D., R.H.H. Miler, J.R. Wright and C.R. Wigganas, 2000. Herd and state means for somatic cell count from dairy herd improvement. J. Dairy Sci., 83: 2782-2788.
14. Booth, J.M., 1998. Antibiotic residues in milk. Impract, 4: 100-109.
15. Currie, D., L. Lynas, G. Kennedy and J. McCaughey, 1998. Evaluation of modified EC four plate method to detect antimicrobial drugs. Food Additives Contaminants, 15: 651-660.
16. Shitandi, A. and O. Sternesjo, 2004. Factors contributing to the occurrence of antimicrobial drug residues in Kenyan milk. J. Food Protection, 67: 399-402.
17. Madigan, M.T., J.M. Martiko and J.M. Parker, 2000. Antibiotic sensitivity. J. Biology of Microorganisms, 44: 3249-3256.
18. Foly, J., J. Buchley and M.F. Murphy, 1974. Commercial testing and product control in the dairy industry. University College Cork.
19. Koenen-Dierick, K., L. Okerman, L. De Zutter, J.M. Degroodt, J. Van Hoof and S. Srebrnik, 1995. A one-plate microbiological screening test for antibiotic residues testing in kidney tissue and meat an alternative to EEC four-plate method. Food Additives and Contaminants, 12: 77-82.
20. Barrow, G.I. and R.K Feltham, 1993. Cowan and Steel manual for the identification for medical bacteria, 3rd ed., Cambridge.
21. Harrigan, W.F. and Margaret, E McCance, 1976. Laboratory methods in food and dairy microbiology. pp: 25-29. Academic Press. London.
22. Baker, F.J. and M.R. Breach, 1980. Antibiotic susceptibility testing. Medical microbiology techniques. Buffer Worth and Co. Publishers Ltd, pp: 343-350.
23. Elghoul, S.M., 1995. Preservation of raw milk by hydrogen peroxide, M.Sc. Thesis, University of Khartoum, Sudan.
24. El Zubeir, Ibtisam, E.M., P. Kutzer and O.A.O. Elowni, 2006. Frequencies and antibiotic susceptibility patterns of bacteria causing mastitis among cows and their environment in Khartoum State. Res. J. Microbiol., 1(2): 101-109.
25. Yagoub, S.O., N.E. Awadlla and Ibtisam, E.M. El Zubeir, 2005. Incidence of some potential pathogens in raw milk in Khartoum North (Sudan) and their susceptibility to antimicrobial agents. J. Animal and Vet. Advances, 4(3): 341-344.
26. IDF, 1994. Pasteurization and heat treatment processes. In: Recommendation for the hygienic manufacture of milk based products. International Dairy Federation, Brucell, Belgium.
27. Adesiyun, A.A., L.A. Webb, H. Romain and J.S. Kaminjolo, 1997. Prevalence and characteristics of strains of *Escherichia coli* isolated from milk and feces of cows on dairy farms in Trinidad. J. Food Protection, 60: 1174-1181.
28. Elmagli, A.A. and Ibtisam, E.M. El Zubeir, 2006. Study on the hygienic quality of pasteurized milk in Khartoum State (Sudan), Res. J. Animal and Vet. Sci., 1(1): 12-17.
29. Mohamed, Ibtisam, E., E.G. Mohamed and E.G. El Owni, O.A.O. 1993. Study on incidence and etiological agent of mastitis of Friesian cattle in Sudan. Proceedings of the Second Scientific Conference, Assuit, Egypt.