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Full Length Research Paper

Factors determining the load of *Staphylococci* species from raw bovine milk in Khartoum State, Khartoum North, Sudan

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In this study, 644 raw milk samples were collected from various milk sources of Khartoum state during May 2003 till April 2004. Using the API kits, both coagulase positive and negative *Staphylococci* (CPS and CNS) were identified. Most of the CPS isolates were *Staphylococcus aureus*, while the majority of CNS were *Staphylococcus epidermidis*. 23.8% of the samples were found to be positive to both CPS and CNS while 33.7% of the samples were negative to both CPS and CNS. Forty one percent of CPS isolates was found to be *S. aureus* and 39% of CNS were *S. epidermidis*. The coagulase positive *Staphylococci* (CPS) were determined in 45% of the tested samples while coagulase negative *Staphylococci* (CNS) were found in 44.7% of them, of which 16.5% exceeded the recommended bacterial safety limits for CPS. The highest of the samples that exceeded this limit was found in vendor milk samples (19.4%) while the market milk was 18.4%.

Key words: Staphylococci, bovine, raw, milk, Sudan.

INTRODUCTION

According to their reaction with Coagulase test, *Staphylococcus* spp are divided into coagulase positive (CPS) which includes the pathogenic *Staphylococcus aureus* (S. aureus) and coagulase negative (CNS) which includes the pathogenic and non pathogenic species of the genus *Staphylococci* (Devriese, 1990).

The infected mammary gland is the primary and most important reservoir of *S. aureus* (Jerry, 2003). Davidson (1961) isolated *S. aureus* from the teat skin, udder skin, nose lips, sacral regions and belly of cows. *S. aureus* was also isolated from teat skin of healthy cattle without being shedding in milk (Davidson, 1961).

API system was evaluated as a means of identifying the species of bovine strain of several groups of micro-organisms isolated from milk samples (Bruce et al., 1983) with similar values reported in human isolates (Gemmel and Dowson, 1982).

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Bran et al. (1978) found the API STAPH identification system to be more accurate in identifying *S. aureus* (93.3%) than non *S. aureus*. Gaery et al. (1989) found the overall accuracy of the API STAPH identification system as 80.9%, when 188 strains of *Staphylococci* were tested, the accuracy among CNS was 86%. Wesley and Jana (1982) reported a high degree of congruence > 90% between the API system and the conventional methods for most species.

Importance of *Staphylococci*

Food or water contaminated with infectious organisms or toxins secreted by such organisms act as a source of food poisoning (Haeghebaert et al., 2003). Bacteria are the leading cause of food born diseases; they cause diseases through the pathogenesis of the organisms in host cells. The pathogenesis of the bacteria causing food born poisoning depends on their capacity to produce toxins after ingestion or before (Haeghebaert et al., 2003).

Table 1. Number and percentage of milk samples from three regions of Khartoum State during summer and winter according to the source.

	Summer				Winter			
	Kh.	**Kh.N.**	**Omd.**	**Total**	**Kh.**	**Kh.N.**	**Omd.**	**Total**
Ind.	5	12	17	34	33	19	13	65
	07.9%	22.2%	27.9%	19.1%	24.8%	09.3%	10.1%	13.9%
Bulk	13	15	19	47	35	109	53	197
	20.6%	23.8%	31.1%	26.4%	26.3%	53.4%	41.1%	42.3%
Vendor	20	11	15	46	42	51	16	109
	31.8%	20.4%	24.6%	25.8%	31.6%	25.0%	12.4%	23.4%
Market	25	16	10	51	23	25	47	95
	39.7%	29.6%	16.4%	28.7%	17.3%	12.3%	36.4%	10.4%
Total	63	54	61	178	133	204	129	466

Kh = Khartoum; Kh.N.= Khartoum North; Omd. = Omdurman; Ind. = Individual.

Some *S. aureus* strains can produce staphylococcal enterotoxins and are the causative agents of Staphylococcal food poisoning (Yves et al., 2003). Although their contamination can be readily avoided by heating of milk, nevertheless, it remains a major cause of food-born diseases because it can contaminate milk after milking and during processing. About 10% of *Staphylococci* causing mastitis in cows produce enterotoxins which are heat stable and may cause food toxicity in man (I.D.F, 1980). *S. aureus* is the most important pathogen of CPS due to its combination of toxin mediated virulence, invasiveness and antibiotics resistance (Yves et al., 2003).

Among coagulase negative species, Jay (1986) and Bautista et al. (1988) reported that *Staphylococcus Cohnii, Staphylococcus xylosus and Staphylococcus haemolyticus* can produce also one or several staphylococcal enterotoxins. Coagulase positive *Staphylococcus Intermedius* have been shown to produce enterotoxins. Kambaty et al. (1994) reported *S. Intermedius* as the only non-*S. aureus* species that has been clearly involved in Staphylococcal food poisoning outbreaks. Ibrahim (1973) showed that an out break of Staphylococcal enterotoxins poisoning in Khartoum North was due to milk purchased from milk vendors.

In Sudan, the traditional ways of milking and distribution of milk are still in use. The majority of the dairy farms do not use milking machines. No system of milk cooling is applied. Traditional ways using donkeys or cars (vendors) are used to distribute milk to consumers which can take more than five hours after milking.

This study was conducted to achieve the following objectives: 1) Enumerate the *Staphylococci* of raw milk produced and sold in the three regions of Khartoum State. 2) Study the effect of seasons on the number of *Staphylococci* in milk. 3) Isolate some of the most important CPS and CNS that can affect human health and milk quality. 4) Propose some quality limits of acceptable raw milk which can be adopted by the Ministry of Agriculture and Animal Recourses of Khartoum state.

MATERIALS AND METHODS

Collection of milk samples

The study was conducted in Khartoum State, using stratified random sampling for the dairy farms. The state was divided into three regions; Khartoum, Khartoum North and Omdorman. Random samples were collected from each region (Table 1).

The samples were collected in sterile Glass bottles either directly from the udder in cases of individual cows or from the milk bulk tanks or milk containers from milk markets and milk vendors. Samples were then kept in an ice box and transported directly to the laboratory at Shambat which is usually ranging between half an hour to one hour in the icebox.

Enumeration and isolation of *Staphylococci*

The test steps to enumerate *Staphylococci* were performed according to (ISO/DIS, 1997). Five decimal dilutions were prepared by adding 10 ml of the milk sample to 90 ml of peptone water in the first glass bottle resulting in 10^1 dilution, the process was continued till a dilution of 10^5 is obtained. For each dilution to be plated, aseptically, 1 ml suspension was spread on 3 plates of Baird Parker Agar medium (0.4, 0.3 and 0.3 ml) of agar plate usi and then incubated for 48 h at 35°C. Plates containing 20 - 200 colonies with appearance of coagulase positive *Staphylococci* were selected. Five to seven colonies from each plate were selected and inoculated into 5 ml brain heart infusion broth and then incubated for 24 h at 37°C. 0.1 ml of brain heart infusion broth was added to a test tube containing 0.3 ml of rabbit plasma. The tubes were incubated for 4 - 6 h at 37°C.

The Baird Parker Agar medium was prepared according to the manufacture instruction (bioMérieux sa). Bacto EY tellurite enrichment (egg yolk emulsion and 3 ml of 3.5% aqueous solution of potassium tellurite) were added and mixed well (avoiding bubbles). The isolates were classified as CPS or CNS using coagulase test.

API STAPH kits

It was prepared and performed according to the manufacturer

Table 2. Seasonal CPS count in the three regions of Khartoum state.

	Khartoum			Khartoum North			Omdurman		
Range	**Winter**	**Summer**	**Total**	**Winter**	**Summer**	**Total**	**Winter**	**Summer**	**Total**
Negative	44	75	119	30	105	135	37	65	102
(%)	69.8	56.4	60.7	55.6	51.5	52.3	60.7	50.4	53.7
< 100	01	05	06	05	11	16	03	12	15
(%)	01.6	3.8	3.1	09.3	05.4	6.2	04.9	9.3	7.9
100 - >500	04	19	23	05	19	24	04	11	15
(%)	06.3	14.3	11.7	09.3	09.3	9.3	06.6	8.5	7.9
500 - $<10^3$	08	16	24	04	23	16	08	16	24
(%)	12.7	12.0	12.2	07.4	11.3	06.2	13.1	12.4	12.6
10^3 - 10^5	06	12	18	10	39	49	09	25	34
(%)	09.6	9.0	9.2	18.5	19.1	19.0	14.7	19.4	17.9
Total	063	133	196	054	204	258	061	129	190

At 0.05 level of significance there was no significant) difference between Khartoum North and Omdurman. The difference was significant between Khartoum and Khartoum North, Khartoum and Omdurman. The count was significantly ($P < 0.05$) higher during summer than winter.

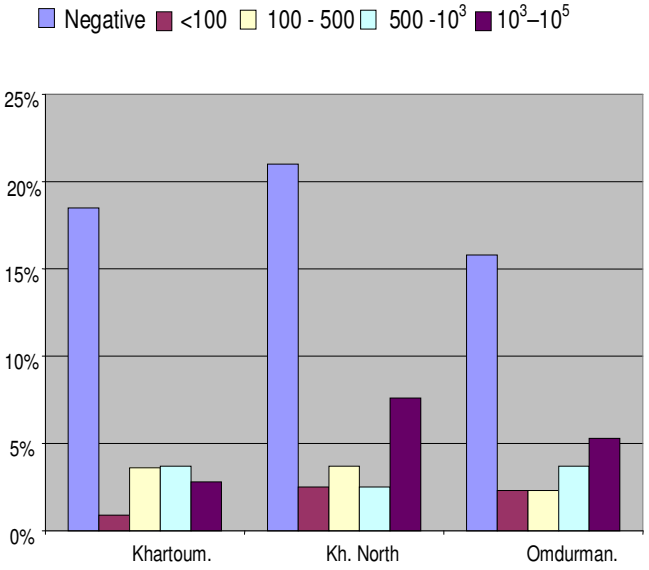

Figure 1. The % of CPS counts in the three regions of the state.

manual API STAPH REF 20 500 (identification system for *Staphylococci*, micro cocci and related genera bioMérieux sa).

The API strip system combining about 20 miniaturized biochemical tests many of which are derived from the conventional methods (Wesley and Jana, 1982). API system is particularly advantageous in providing preformed strips containing the test substrates and made available necessary reagents and also in most cases reaction could be interpreted after incubation at 37°C for 24 h (Bran et al., 1978).

Statistical analysis

Microsoft excel 2003 and the spss for windows version 11 were used for data analysis. t-test was selected to calculate the significance levels. Descriptive statistics were used.

RESULTS

Enumeration of CPS

The percentage of negative samples to CPS test in Khartoum State in the two seasons was 55.3%, of which 17.2% was in winter and 38.1% in summer. The percentages of samples with CPS count between 10^3 - 10^5 cell/ml were 15.7, 3.9 and 11.8% in Khartoum, Khartoum North and Omdorman, respectively (Table 2 and Figure 1). Statistically at 0.05 level, there was no significant difference between the three regions. However, the difference between the two seasons was statistically significant (Table 2).

In Khartoum state, the percentage of samples of negative counts during the two seasons were 65.7, 57.8, 46.9 and 51.3%, respectively in individual, bulk, vendor and market milk. In winter, the percentages of such counts were 73.5, 63.8, 58.6 and 56.9%, respectively and in summer, the percentages were 61.5, 56.3, 50.5 and 48.4% in individual, bulk, vendor and market milk, respectively (Table 3). A count between 10^3 - 10^5 cell/ml in Khartoum state was found to be 17.2, 19.3, 19.4 and 18.5% in individual, bulk, vendor and market milk, respectively during the two seasons, while in winter, the percentages of the same counts were 14.7, 17.0, 17.4 and 17.6%, in summer these counts were 18.5, 19.8, 20.2 and 18.9%, in individual, bulk, vendor and market milk, respectively (Table 3). Statistically, there was a significant correlation (at 0.01 levels) between individual, farm bulk milk, vendor and market milk counts (Table 4 and Figure 2).

The CPS and CNS

23.8% of the samples were positive to both CPS and

Table 3. Seasonal Total CPS count in the State, according to the source.

	Summer					Winter				
Range	**Ind.**	**Bulk**	**Vendor**	**Market**	**Total**	**Ind.**	**Bulk**	**Vendor**	**Market**	**Total**
Negative	025	030	027	029	111	040	111	048	046	245
(%)	73.5	63.8	58.6	56.9	62.4	61.5	56.3	50.5	48.4	52.6
< 100	01	02	03	03	09	03	11	08	06	28
(%)	2.9	4.3	6.5	5.9	5.1	4.6	5.6	7.3	6.3	6.0
100 - < 500	01	03	04	05	13	04	14	18	13	49
(%)	2.9	6.5	10.9	7.8	7.3	6.2	7.1	6.5	13.7	10.5
500 - $<10^3$	02	04	05	04	15	06	22	13	12	53
(%)	5.9	8.5	10.9	7.8	8.4	9.2	11.2	11.9	12.6	11.4
10^3 - 10^5	05	08	08	09	30	12	39	22	18	91
(%)	14.7	17.0	17.4	17.6	16.9	18.5	19.8	20.2	18.9	19.5
Total	034	047	046	051	178	065	197	109	095	466

Table 4. Correlations of CPS between the individual, Bulk, Vendor and market milk.

		Individual	**Bulk**	**Vendor**	**Market**
Individual	Pearson correlation	1	0.962(**)	0.960(**)	0.995(**)
	Sig. (2-tailed)	.	0.002	0.002	0.000
	N	6	6	6	6
Bulk	Pearson correlation	0.962(**)	1	0.942(**)	0.972(**)
	Sig. (2-tailed)	0.002	.	0.005	0.001
	N	6	6	6	6
Vendor	Pearson correlation	0.960(**)	0.942(**)	1	0.973(**)
	Sig. (2-tailed)	0.002	0.005	.	0.001
	N	6	6	6	6
Market	Pearson correlation	0.995(**)	0.972(**)	0.973(**)	1
	Sig. (2-tailed)	0.000	0.001	0.001	.
	N	6	6	6	6

** Correlation is significant at the 0.01 level (2-tailed).

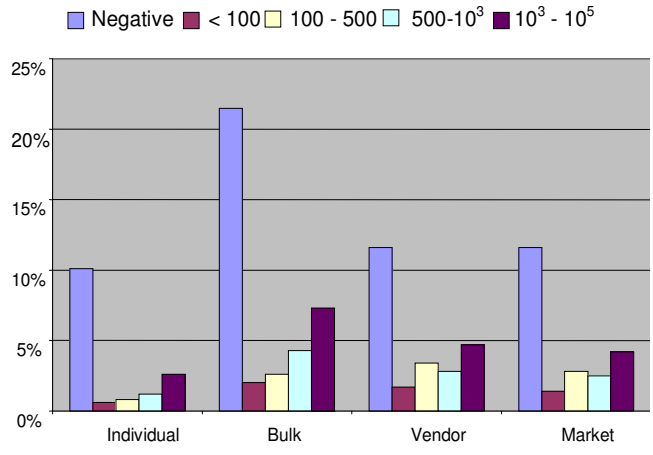

Figure 2. The % of the CPS counts at different sources.

CNS during the two seasons, 33.7% of the samples were negative to both CPS and CNS during the two seasons.

Table 5. The Number and percentage of CPS and CNS milk samples in Khartoum state.

		CPS		
CNS		**Positive**	**Negative**	**Total**
	Positive	153	139	292
	(%)	23.8	21.5	45.3
	negative	135	217	352
	(%)	21.0	33.7	54.7
	Total	288	356	644
	(%)	44.7	55.3	

45.3% of the whole samples were positive to CNS and 44.7% were positive to CPS (Table 5).

Identification of the CPS and CNS isolates

Using the API STAPH system, 275 isolates of the total

Table 6. The percentages of CPS and CNS isolated from the vendor and market milk from Khartoum state.

Species	Kh..	(%)	Kh.N	(%)	Omd	(%)	Total	(%)
S. aureus	26	9.50	55	020	32	11.6	113	41.10
S. intermedius	01	0.40	01	00.4	02	00.7	004	01.50
S. epidermidis	29	10.5	36	13.1	44	016	109	39.60
S. lentus	02	0.70	03	01.1	01	00.4	006	02.20
S. capitis	01	0.40	03	01.1	00	00.0	004	01.50
S.Simulans	00	1.50	02	00.7	02	00.7	004	01.50
S. caprae	00	0.00	00	00.0	02	00.7	002	00.70
S. xylosus	02	0.70	01	00.4	02	0.70	005	01.80
S. chromogens	02	0.70	03	01.1	00	00.0	005	01.80
S. sciuri	03	1.10	00	00.0	00	0.00	003	01.10
S. hyicus	05	1.80	02	00.7	02	0.70	009	03.30
S. hominis	01	0.40	02	00.7	00	0.00	003	01.10
Micrococcus spp	04	1.50	02	00.7	02	0.70	008	02.90

samples were identified of which 41.1% were *S. aureus* whereas 39.6% of the CNS isolates were *S. epidermidis* (Table 6). Other isolates includes *S. intermedius, S. lentus, S. capitis*, *S. xylosus*, *S. hyicus*, *S. caprae*, *S. sciuri*, *S. simulan*, *S. hominis* and *Micrococcus* spp.

DISCUSSION

The high prevalence of *Staphylococci* could be due to the fact that the herdsmen milk wet udders, using same cloth for washing the udders, the cloth was washed in the same water bucket and milkers usually clean their hands between milking by passing the hand through their cloths. Milking is usually performed inside the same shade for there were no separate milking areas. All these unhygienic conditions contribute a lot to the bacterial count before it reaches the milking buckets. The chain may last for five hours till milk reaches the consumer.

Due to all these factors which may affect the count and elevate it at any level, it was expected that milk would have a moderate to high bacterial count.

CPS were primarily mastitis pathogens, their presence in the milk is favored by udder infection, the hygienic measure before, during and after milking and the environmental conditions (Anderson, 1982).

During this study in Khartoum state, percentage of the samples which were positive to CPS were higher in summer (47.4%) compared to 37.6% in winter. Khartoum north was the region with higher percentage of positive samples in winter (44.4%) and Omdurman has the highest percentage during summer (49.6%) while Khartoum region has the lowest percentage during the two seasons (30.2%) in winter and (43.6%) in the summer. The difference between the CPS positive sample in Khartoum in one side and Khartoum north and Omdurman on the other side was significant at 0.05, but the difference between Khartoum north and Omdurman was insignificant. This may be partially due to the better hygienic practices in most of the Khartoum region farms, since most of the farmers were educated retired government employees. Also, they can use the mastitis medicine properly and can educate their farmers. The percentage of CPS positive samples in vendor milk was the highest (53.1%), then market milk (48.8%), then the bulk tank farm milk, which was 42.2%, since the number of farms from which milk is collected varies between vendor and market milk, while the milk in the farms bulk tank is almost collected from the apparently mastitis free cows, but addition of milk of the neighboring farms to farm bulk milk is practiced in some farms. Nada (2000) found these percentages to be 20% in vendor milk and 25% in farm bulk milk. These differences may be due to the differences in sample size between the two studies.

The bacterial safety limits for the CPS was recommended by Bruce (2003) not to exceed 10^3. In this study, percentage of samples which exceeded this limit was 16.5% in the state, Khartoum north has the highest percentage followed by Omdurman then Khartoum region. The vendor milk which exceeds this limit was the highest (19.4%) then market milk (18.4%), Bulk (18%) and then the individual which was 17.1%. These higher percentages of counts reflect the poor hygienic measure at milking time and during distribution.

Statistically, there is significant correlation at 0.01 significance level between the Individual, farm bulk tank, market and vendor milk; this is again due to subclinical mastitis, milking of unhygienic udder, poor sanitation of milkers distributors and milk containers.

In conclusion, it was found that most of the raw milk sold in Khartoum State is of low hygienic quality, so, the Ministry of Agriculture and Animal Resources of Khartoum State should enforce all the regulations needed for producing and purchasing raw milk with acceptable

hygienic quality. Some of these regulations should include: 1) Small producing units should be grouped together to facilitate veterinary services and direct supervision. 2) Khartoum State should establish collection centers to receive and cool the milk immediately after production. 3) The practice of selling raw milk through the pick-up cars and carriages without least hygienic measures should be stopped. Milk must be treated before reaching the consumers. 4) The presence of *Staphylococci* must be more investigated to determine their impact on public health and their pathogenicity.

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