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Full Length Research Paper

Microbiological Assessment and Identification of Coliforms in Raw Milk from Khartoum State, Sudan

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To determine the most probable number (MPN) of *Coliform* and to identify the *Coliform* bacteria present in the raw milk in the three geographical areas of Khartoum state. Six hundred and forty four raw milk samples were collected during the period between April 2008 to February 2009. The *Coliform* limits in the raw milk accepted internationally are less than 100 cell/ml. About 51.3% of the samples from the milk available for direct consumption in Khartoum state [vendor + (shops) market milk] satisfy this limit. In winter and summer the percentages of milk samples which satisfy this limit were 70.4 and 51.3%, respectively. Vendor milk is more contaminated with *Coliform* bacteria compared to milk from the shops; only 47.8% were in the acceptable limits during winter and 43.7% summer. The difference between winter and summer counts, and the differences between individual, bulk, vendor and shops were statistically significant, at p(0.05). In this study 60.1% of all the raw milk samples in the state were of counts between 0 to <100 cell/ml, but in winter the percentage (76.9%) was higher than summer (53.6%). Statistically there was a significant difference between the two seasons in the state, but the differences between these three areas were statistically insignificant. The majority of the *Coliform* isolates from the raw milk consumed in Khartoum state were *Escherichia coli* 32%, Enterobacter species 29.2%, Klebsiella species 19.4%, *Serratia* species 11.1% and Citrobacter 1.0%, in addition to some Enterobacteriaceae.

Key words: *Coliform*, Sudan, count, isolation, most probable number (MPN).

INTRODUCTION

Milk has played a major contribution in the human diet in many different countries across the world. It is not surprising therefore, that over many years considerable attention has been paid to improve the quality of milk particularly the hygienic quality. The chain of people involved in dairy production in such under-developed countries extend from milk production farm (farmers, farm workers and veterinarians), transportation to milk market or to small vendor to reach the final consumers either through milk vendors or from shops. The total amount of raw milk produced in the State as estimated by Ministry of Agriculture and Animal Recourses in 2007 was 438,726 tons of milk, still 95% of this milk is distributed as raw milk to the consumer. One of the requirements in the production of high quality raw milk is maintaining acceptable bacterial counts which meet the official milk quality standards. The presence of bacteria in milk can cause some reduction in the raw milk quality and certain bacteria contaminants with their associated enzymes and toxins may even survive pasteurization and create health hazards (Oliver et al., 2005). The objectives of this study include:

1) Evaluate the effect of time on the *Coliform* count of raw milk produced and sold in the three regions of Khartoum State.
2) Study the effect of seasons on the *Coliform* counts of raw milk.
3) Isolate some of the most important *Coliform* bacteria that can affect human health and milk hygiene.

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4) Propose some quality limits for Coliform in raw milk which can be adopted by the Ministry of Agriculture and Animal Resources of Khartoum State.

Coliform bacteria

The genera *Escherichia, Klebsiella, Enterobacter, Serratia,* and *Citrobacter* (collectively called the *Coliform bacilli*) and *Proteus* some of them are opportunistic pathogens responsible for a wide range of infections, but many species are members of the normal intestinal flora. *Escherichia coli* (*E. coli*) is the most commonly isolated organism in the clinical laboratory (Baron, 1996). *Coliform*s are almost always found in raw milk but with good methods of production number of *Coliform*s can be kept very low (Boor et al., 1998). The presence of these organisms in milk and milk products is an indication of unsanitary production and\or improper handling of either milk or milk utensils (El-zubeir and Ahmed, 2007). Milking udder with sub-clinical mastitis and wet environment lead to contamination of bulk tank milk and hence raw milk reaches the consumers with elevated Coliform count (FAO, 2008; Zadoks et al., 2007). Kagki et al. (2007) showed that in addition to faecal contamination, other factors such as milking wet udders, inadequate cooling of milk and udder infection are the main sources of *Coliform* in bulk milk. College of Agriculture and Life Science (2001) asserted that *Coliform*s are associated with fecal and environmental contamination. *Coliform* count of less than 100 cell/ml is considered acceptable, but count of less than 10 cell/ml is achievable and desirable (Boor et al., 1998). *Coliform* count above 500 cell/ml indicates poor hygiene either during equipment cleaning or between milking with common contaminants such as bedding, manure, soil or water (Murphy and Boor, 2003).

Bulk milk *Coliform* bacteria are used as indicator of hygienic condition during handling and processing of milk and milk products (College of Agric and life Sciences, 2001). In addition to the use of solid media from which the *Coliform* density can be counted the use of liquid media which can be worked out by the most probable number (MPN) can be used (Messer and Dufour, 1998). This MPN method has been shown to produce satisfactory results with naturally-contaminated foods for the detection of *Coliform*, faecal *Coliform* and *Enterococci* (APHA, 1985). The method of *Coliform* counting has two stages; the presumptive test and the confirmed test. The presumptive tests are designed to grow the target bacteria and the media in the confirmed test are used to validate the growth of target bacteria in the presumptive test (Pettibone, 1992; ISO/CD, 1997). The ISO/CD (1997) suggested that the use of “brilliant green” as a liquid media for the enumeration of *Coliform* using the MPN method allows large quantities of product to be examined. The “brilliant green” lactose bile broth was also an acceptable test to be performed on milk samples for the confirmation of *Coliform* count (Andreus, 1997).

MATERIALS AND METHODS

In this study 644 raw milk samples were collected from the three main geographical regions of Khartoum state during the period between April 2008 to February 2009 (Table 1). The samples were collected at two different levels during summer and winter:

1) The farm level; milk from individual cows (individual) and farm bulk tank (bulk) were collected during winter and summer (Table 1).
2) The market level; from the main milk markets (market) that is shops and small vendors (vendor) during winter and summer (Table 1).

Sample collection and preparation

About 50 ml of milk were collected in sterile glass bottle either directly from the udder in cases of individual cows or from the milk tank or milk containers. Samples were then kept in an ice box and transported directly to the laboratory at the Faculty of Vertinary Science in Khartoum University.

Total *Coliform*s count

Presumptive test

According to ISO/CD (1997) and Andreus (1997), the Lauryl tryptose broth was used as the media for the presumptive test for total *Coliform*s count. Peptone water was used as a diluent; this result in a dilution of 10^1, 10^2, 10^3, 10^4 and 10^5. A Durham tube was inserted into each Lauryl Tryptose tube. 1 ml of each dilution was pipetted into 3 Lauryl Tryptose tubes. All tubes were incubated at 35 to 37°C for 48 h and then examined for gas formation in the Durham tubes.

Confirmed test for Coliforms

Each positive (gassing) Lauryl Tryptose tube was gently agitated and a loopful of suspension was transferred to tube of “brilliant green” bile broth. All the tubes were incubated at 35 to 37°C; any gas formation in Durham’s tubes with slight turbidity in the media was regarded as positive confirmed test. Results were interpreted using the MPN tables based on combination of confirmed gassing of Lauryl Tryptose broth tubes for three consecutive dilutions (ISO/CD, 1997).

Confirmed test for E. coli

According to ISO/CD (1997), Levine Eosin methylene blue (L-EMB) agar was used. The plates were streaked with a loopful of suspension from confirmed positive brilliant green bile broth culture. Plates were incubated at 35°C for 18 to 24 h. Discrete dark centered nucleated colonies with or without metalic sheen were regarded as a positive test. Two colonies or more were picked from each (L-EMB) agar plate and transferred to nutrient agar slants for morphological examination of all gram negative, short rods or cocci were identified using analytical profile index test kits (API). The API test kits used were API 20 E Kit and rapid API 20 E Kit, which were prepared and performed according to the manufacture manual (bio Merieux) (system for the identification of enterobacteriaceae).

RESULTS

The percentage of samples with count less than 100

Table 1. Number and percentage of milk samples according to areas and seasons.

Level	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

Table 2. Seasonal *coliform* count in the three regions of Khartoum State.

Range	Khartoum			Khartoum North			Omdurman		
	Winter	Summer	Total	Winter	Summer	Total	Winter	Summer	Total
Negative	37	60	97	24	73	97	38	43	81
%	58.7	45.1	49.5	44.4	35.8	37.6	62.4	33.3	42.6
<100	10	15	25	13	39	52	15	20	35
%	15.8	11.3	12.8	24.2	19.1	20.2	24.6	15.5	18.4
100-300	5	6	11	07	29	36	04	05	09
%	08.0	04.5	05.6	12.9	14.2	14.0	06.5	03.9	04.7
300-600	0	9	09	02	8	10	00	11	11
%	0.00	06.8	04.6	03.7	03.9	03.9	0.00	08.5	05.8
1100	5	13	18	02	17	19	01	17	18
%	08.0	09.8	09.1	03.7	08.3	09.3	01.6	13.2	09.5
> 1100	06	30	36	06	38	44	03	33	36
%	09.5	22.5	18.4	11.1	18.6	17.1	04.9	25.6	19.0
Total	063	133	196	054	204	258	061	129	190

At 0.05 level of significance there were no significant differences between Khartoum, Khartoum North and Omdurman. At 0.05 level of significance, there is a significant difference between the two seasons.

cell/ml in Khartoum state during the two seasons were 60.1%. In winter they were 76.9% while in summer they were 53.6% (Table 2). A count of more than 1100 cell/ml was reported in 18.0% of the samples in the state during the two seasons; in winter and summer the percentages of this count were 08.4% from winter samples and 21.7% from summer samples (Table 2). The percentage of samples with counts less than 100 cell/ml in Khartoum region was 62.3% during the two seasons, during winter and summer the percentages of counts were 74.5 and 56.4% respectively (Table 2). The percentage of the same count in Khartoum North during the two seasons was 57.8%. During winter and summer the percentages of counts of less than 100 cell/ml were 68.6 and 54.9% respectively (Table 2). The percentage of the same count in Omdurman during the two seasons was 61%. During

winter and summer the percentages of this count were 87.0 and 48.8%, respectively (Table 2). A count of more than 1100 cell/ml was reported in 18.0% in Khartoum state, 18.4% in Khartoum region, 17.1% in Khartoum North and 18.9% in Omdurman during the two seasons; in winter and summer the percentages of the counts were 08.4 and 21.7% in Khartoum state respectively (Table 2). Statistically at P (0.05) there were no significant differences between the three geographical regions of the state. The difference between the two seasons was statistically significant P(0.01). In Khartoum state the percentages of counts of less than 100 cell/ml were 81.8, 62.1, 50.6 and 52.1% during the two seasons in individual cow's milk, farm bulk tank milk, vendor and market milk respectively. During summer the counts were 76.9, 57.9, 43.1 and 41.0% in individual cow's milk, farm bulk tank milk, vendor and market milk, in winter these percentages were 91.2, 80.4, 68.1 and 72.5% respectively (Charts 2 and 3).

A count of more than 1100 cell/ml was found to be 0.00, 13.6, 21.8 and 19.2% during the two seasons. In winter the counts of more than 1100 cell/ml were 0.00, 02.2, 17.0 and 11.8% in individual cow's milk, bulk tank milk, vendor and market milk respectively; during summer the counts were 0.00, 16.2, 23.9 and 23.2% respectively (Table 3). Statistically there was strong correlation between the counts in bulk tank milk and the vendor milk at P (0.01). The farm bulk milk and market milk correlated at P (0.05) and there was no correlation between the individual cow milk and farm bulk milk (Table 3). Using the (L-EMB) agar the percentage of fecal *Coliform* isolated from the positive *Coliform* tubes were 57.8%. 300 *Coliform* isolates were identified from the samples of vendor and market milk (Table 4) (Chart 1). 32% were *E. coli*, 13.6% were *Enterobacter cloacae* and 11.3% were *Enterobacter aerogenes.* Other *Enterobacter* species included *Enterobacter gergoviae, Enterobacter sakazaki* and *Enterobacter cancerogenus*. *Klebsiella planticola* were 10.7% in addition to *Klebsiella ornithinolytica* and *Klebsiella pneumoniae*. *Serratia odorifera* were 2.4%, *Serratia liquefaciens* and *Serratia marcescens*. *Vibrio* species included *Vibrio fluvialis* and *Vibrio cholerae*, other gram negative organisms included *Citrobacter koseri, Citrobacter freundii, Proteus vulgaris, Proteus penneri, Pseudomonas aerugenosa, Ewingella Americana* and *Pantoea* species (Chart 4).

DISCUSSION

The acceptable limits of *Coliform* counts in milk should be less than 100 cell/ml (Shojaei and Yadollahi, 2008; Douglas, 2003; Boor et al., 1998) but some prefer a count of 50 cell/ml (College of Agriculture and Life Science, 2001). In this study 60.1% of all the samples in the state were of counts between 0 to <100 cell/ml, but in winter the percentage of such count was higher (76.9%) and in summer It was 53.6%. This was in agreement with Muhammed et al. (2009) and Akhmedov et al. (1976) who showed that *Coliform* count was higher in hot season, this showed that the majority of the raw milk in the state was within the accepted limits. The percentage of acceptable *Coliform* counts in Khartoum region was the best (62.3%), followed by Omdurman (61%) then Khartoum North (57.8%) with better acceptable percentage during winter in all the three areas, this was in line with Gouranga (2008) who reported better counts in winter if compared to summer and autumn. *Coliform* counts of more than 100 cell/ml suggests poor hygienic practices (Jayarao and Wolfgang, 2003). Higher *Coliform* counts were reported in many countries; Khan et al. (2008) reported a count between 300- 400 cell/ml, lower than counts of more than 600 cell/ml reported in the summer market milk. Mutukumiram (1996) calculated a higher rate ranging between 3200 to 23000 cell/ml. Count of 1000 cell/ml was reported by Saitanu et al. (1996); and Shoiaei and Yadollahi (2008) estimated a range between 1000 to 1300 cell/ml. During this study the percentage of the highest count of more than 1100 cell/ml was higher in summer (17.1%) compared to 8.4% during winter. while this count was higher in Omdurman (19.0%), followed by Khartoum (18.4%) then Khartoum North which was 17. 1% but the differences between the *Coliform* counts in the three regions were statistically insignificant. During summer seasons the *Coliform* count were higher in the three regions compared to count in winter and the difference between the two seasons was statistically significant P (0.01).

In vendor milk, the *Coliform* count of more than 600 cell/ml was reported in about 40% of summer market milk compared to about 23% in winter (Chart 2). Count of more than 1100 cell/ml was found to be 21.8% during the two seasons and higher in summer (23.9%) compared to winter (17.0%). This higher count of vendor milk was reported by El Zubeir and Ahmed (2007) and Singh et al. (1975) who reported a count of more than 5600 cell/ml in vendor milk. The percentage of acceptable limits of *Coliform* counts in vendor milk was the lowest in summer, but in winter the percentages of vendor and market milk were almost equal. Statistically, there is no correlation between individual and bulk tank milk, this may be due to the fact that *Coliform*s are associated with fecal and environmental contamination such as bedding, soil, water and inadequate cooling of milk (Douglas, 2003) so it can contaminate the milk through bulk tank container. In this study 76.5% of individual cows' milk were free of *Coliform* in winter and (61.5%) in summer compared to 47.8 and 43.7% in bulk tank milk in the two seasons. These finding were in agreement with Beniwal et al. (1998) for they reported a change in the *Coliform* count of 1000 to 4700 cell/ml and may reach 9000 at the end of the chain, also Shoiaei and Yadollahi (2008) noticed a significant difference in the *Coliform* count of raw milk sold at different shops. Fecal *Coliform* was isolated from 57.8% samples of *Coliform* positive tubes using the EMB agar.

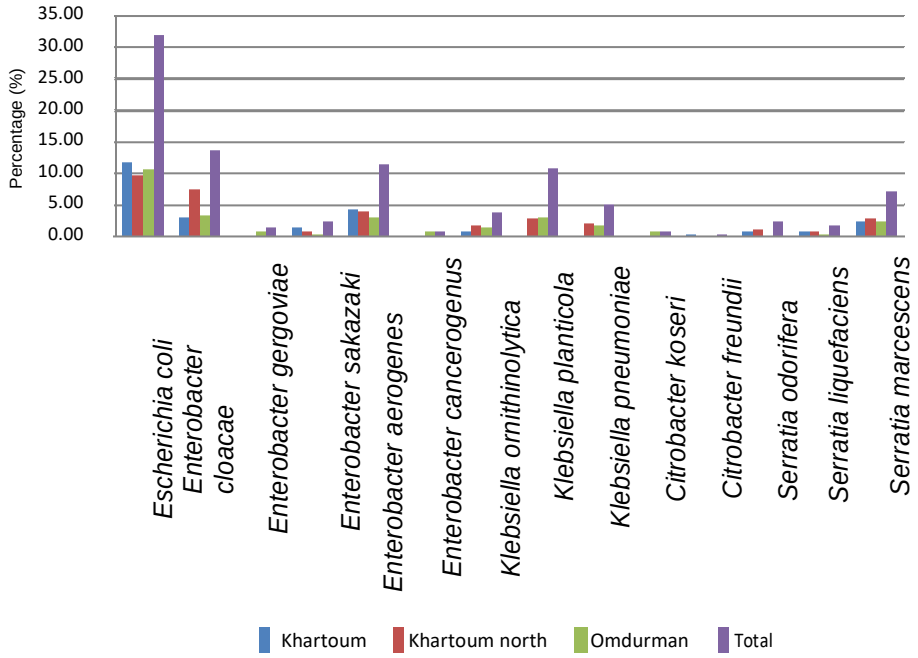

Chart 1. Percentage of *Coliform* organisms isolated from the vendor and market milk from Khartoum State.

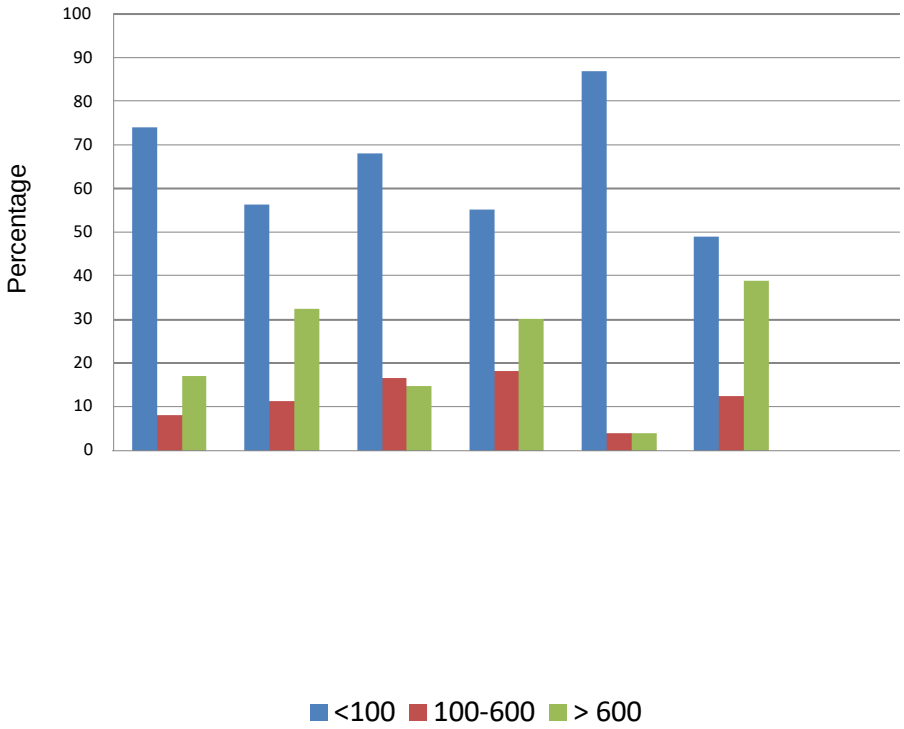

Chart 2. Percentage of *Coliform* count according to season and geographical areas.

These were further identified using the API system. Most of the *Coliforms* isolates were *E. coli* 32%, *Enterobacter* spp. 29.2%, *Klebsiella* spp. 19.4%, *Serratia* spp. 11.1% and *Citrobacter* 1.0%, in addition to some Enterobacteriaceae. These findings were higher than the percentages isolated by Sana et al. (2005); isolated

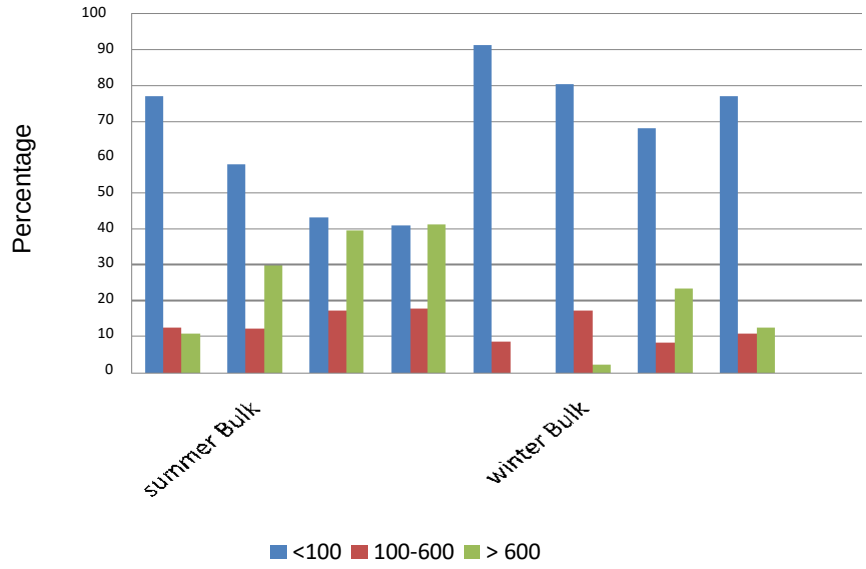

Chart 3. Percentage *coliform* count according to the season and collection levels.

Table 3. Seasonal total *Coliform* count in Khartoum State at different levels of collection.

	Summer					Winter				
Range	**Ind.**	**Bulk**	**Vend.**	**Mark.**	**Total**	**Ind.**	**Bulk**	**Vend.**	**Mark.**	**Total**
Negative	40	86	27	23	176	26	22	22	29	99
%	61.5	43.7	24.8	24.2	37.8	76.5	47.8	46.8	56.9	55.6
1-<100	10	28	20	16	74	05	15	1 10	08	38
%	15.4	14.2	18.3	16.8	15.9	14.7	32.6	21.3	15.6	21.3
100->300	04	15	16	05	40	02	08	03	04	17
%	06.2	7.6	14.7	05.3	08.6	05.9	17.4	06.4	07.8	09.6
300->600	04	09	03	12	28	01	00	01	00	02
%	06.2	04.6	02.7	12.6	06.0	02.9	00.0	02.1	0.00	01.2
1100	07	27	17	17	68	00	00	03	04	07
%	10.7	13.7	15.6	17.9	14.6	00.0	00.0	06.4	07.8	03.9
>1100	0	32	26	22	80	00	01	08	06	15
%	0.0	16.2	23.9	23.2	17.1	00.0	02.2	1 17.0	11.8	08.4
Total	065	197	109	095	466	034	046	047	051	178

E. coli from 14.2% from the raw milk samples, *Enterobacter* spp. 12.8%, *Shigella* spp. 20% and Citrobacter 21.4%. The higher percentage of *E. coli* may be due to the fact that *E. coli* may grow in raw milk and reaches higher number in tropical countries or in the absence of cooling system.

The type of isolates were in agreement with Kagkli (2006) who found *Coliform* present in the milk were *Hafnia alvei*, *S. liquefaciens*, *Yersinia enterocolitica* and *Enterobacter amnigenus*. Jayarao and Wang (1999) confirmed the presence of *E. coli*, *Enterobacter*, *Klebsiella* spp. and *Citrobacter* as major *Coliform* associated with lowering the quality of raw milk.

Conclusions

The lack of knowledge about clean milk production, use

Table 4. Correlations of *Coliform* count between individual, bulk, vendor and market milk in the state correlations.

			Bulk	Vendor	Individual	Market
Spearman's rho	Bulk	Correlation coefficient	1.000	1.000(**)	0.406	0.886(*)
		Sig. (2-tailed)	.	.	0.425	0.019
		N	6	6	6	6
	Vendor	Correlation coefficient	1.000(**)	1.000	0.406	0.886(*)
		Sig. (2-tailed)	.	.	0.425	0.019
		N	6	6	6	6
	Ind.	Correlation coefficient	0.406	0.406	1.000	0.348
		Sig. (2-tailed)	0.425	0.425	.	0.499
		N	6	6	6	6
	Market	Correlation coefficient	0.886(*)	0.886(*)	0.348	1.000
		Sig. (2-tailed)	0.019	0.019	0.499	.
		N	6	6	6	6

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed).

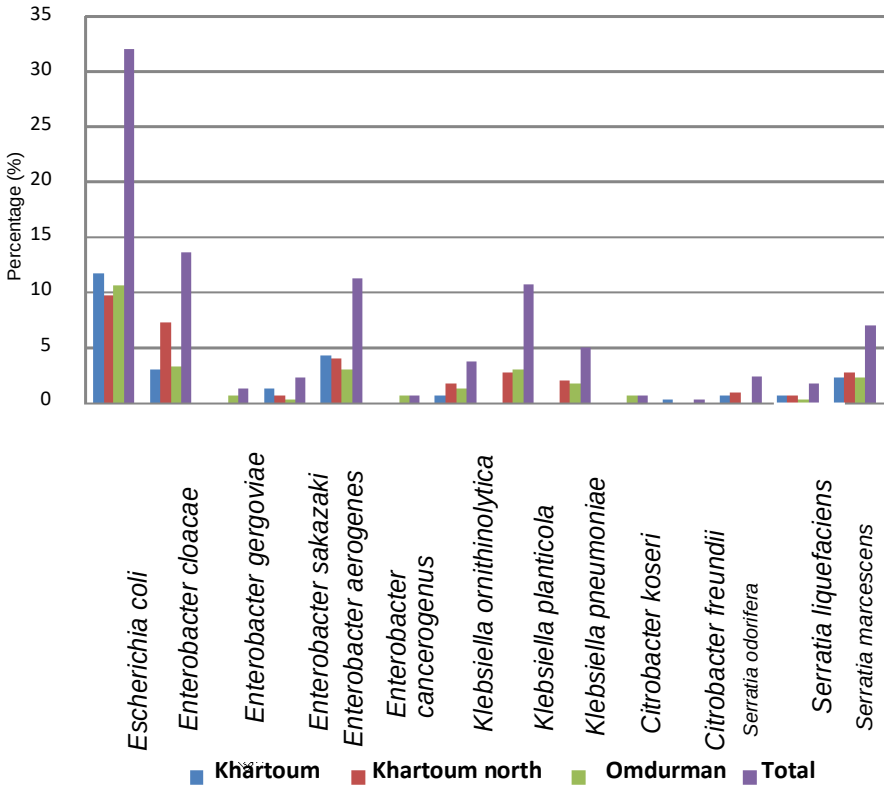

Chart 4. Percentage of *Coliform* organisms isolated from the vendor and market milk from Khartoum State.

of unclean milking equipment and lack of potable water for cleaning purpose were some of the factors which contributed to the poor hygienic quality of raw cows' milk at farms and at collection centers, in the three regions of the state. The level of counts of less than 100 cell/ml is achievable if some better hygienic practices implemented in addition to introduction of a cooling system for the milk in the farm or during the distribution process.

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