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Isolation and Molecular Identification of *Staphylococcus Species* in Cow's Milk Distributed in Khartoum State

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THE AIM of this study was to identify and evaluate the bacteria load and the level of *Staphylococcus Species* and *Staphylococcus aureus* contamination in raw cow´s milk distributed in Khartoum state. One hundred and eighty samples were taken from Khartoum, Omdurman and Khartoum North, 60 samples of raw milk from healthy apparently cows in farms, 60 samples of raw milk vended by donkeys, 60 samples of raw milk vended by cars. Total Viable Bacterial count was done using standard plate count. The samples were detected for the presence of *Staphylococcus Species* using conventional methods, cultured in Baird-Parker and Mannitol agar, Gram Stain, biochemical tests were done. The TVBC showed that the highest bacterial load was detected in the raw milk vended by Donkey (6.90±.03 $\log_{10}$ cfu/ml) vended by cars (6.78±0.12 $\log_{10}$ cfu/ml) then the lowest bacterial load detected in the milk collected from farm (6.63±0.07 $\log_{10}$ cfu/ml). Out of 180 samples of raw milk studied, 130 showed contamination by *Staphylococcus species* corresponding to 72.2% of the samples being contaminated and out of 180 samples of raw cow´s milk 80 was contaminated with *staphylococcus aureus* corresponding to 44.4% of the samples.The isolated *Staphylococcus species* was confirmed further by using the Polymerase Chain Reaction (PCR) targeting the partial sequence of 16s rRNA gene. Sequencing identified *Staphylococcus aureus*, *staphylococcus epidermidis, Staphylococcus hominies* and *Staphylococcus simulans.* The results showed a high level of contamination by *Staphylococcus Spp. and staphylococcus aureus* in raw cow´s milk that distributed in Khartoum state.

Keywords: Cow's milk, Pathogenic, *Staphylococcus species,* Contamination.

Introduction

Milk is ideal environment for bacterial growth for its enriched ingredients, so the isolation of some pathogens is very important. The analysis of milk regarding pathogenic microorganisms is a clear indicator of hygienic quality which influences the dairy production [1].

The presence of the contaminating micro-organisms in the milk, produced by small scale dairy farmers in Shahrekord Southwest Iran where milking was done by hands showed high percentage of *staphylococcus* contamination [2]. Most of the low quality milk due to high degree of poor hygiene practice during milking, transporting, distributing, processing and handling [3].

The presence of *Staphylococcus aureus* in raw milk generally comes from cows with subclinical mastitis, from workers, handlers or from deficient hygiene so the high levels of contamination can be reached quickly under favourable conditions and their presence in foods can be high risk to

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human health, causing a public health problem, as these bacteria can cause food intoxication [4].

Staphylococcus aureus is an important food-borne pathogen, of both humans and animals which causes a variety of diseases with severity of slight skin infection to highly severe diseases like septicaemia and pneumonia. *Staphylococcus aureus* is present on the skin and mucosa of food-producing animal reservoirs, such as ruminants [5], so milk and milk product contaminated with *staphylococcus spp.* may cause food poisoning and pose a threat to public health [6].

From a food safety point of view, the *staphylococcal* enterotoxin (SE) production is the most crucial problem which leads to the *staphylococcal* food poisoning outbreaks in humans as the third most common food intoxication in the world [7].

Analysed samples of raw cow´s milk for pathogens *Staphylococcus aureus* and he reported that the contamination by *staphylococcus aureus* may directly affect the human health causing public, and can also affect dairy industry [8].

Therefore, the key objectives of this study was to assess and to evaluate raw cow's milk vended with deferent channelled in Khartoum State and to isolate and identify *staphylococcus aureus* and other *staphylococcus spp.,* using molecular technique.

Material and Methods

A total of 180 samples of milk (dairy farms, milk vended by donkey and milk vended by cars) were collected from 3 localities Omdurman, Khartoum and Khartoum North in sterile containers and brought in porTable insulated cold boxes at 4 °C to the laboratory of the Collage of Veterinary Medicine, Sudan University of Science and Technology during the period of July 2016 to September 2017.

Total Viable Bacterial Count (TVBC)

Serial dilution were done from10^{-1} to 10^{-8}, from the diluent 10^{-4} and the diluent 10^{-5} 1 ml was taken and cultured on nutrient agar medium and incubated for 24 hours at 37° C then the total viable bacterial count (TVBC) were done, [9].

Isolation and Identification of Bacteria

For the isolation and identification of bacteria, using conventional methods for *staphylococcus spp.* was determined by culture on mannitol salt agar (selective medium for staphylococcus), the plates were incubated at 37 °C for 24 h [10]. The number of yellow colonies in un-crowded plates was counted. Pure organisms were obtained by sub-culturing of isolated typical colony on the nutrient agar medium for 24 hours at 37 ° C.

The organisms were identified on the basis of their cultural, morphological, Gram stain and various biochemical characteristics Oxidase, Catalase, Oxidation–fermentation tests and Coagulase test for *staphylococcus aureus*.

The confirmation of isolated *staphylococcus species* was done by molecular technique using polymerase chain reaction (PCR) at research laboratory, Sudan University of Science and Technology and sequencing of PCR products and BLAST of 16S r RNA gene.

Genetic Identification.

The isolates of *Staphylococcus species* were cultured on Baird Parker agar, supplemented with egg yolk tellurite, followed by sub culturing of a single colony in nutrient agar medium for 24 hours at 37 ° C.

DNA Extraction

50 µL of distilled water was transferred to Eppendorf tube, pure colonies (n.3) of isolated *Staphylococcus spp.,* transferred to the Eppendorf tube, 5 µL of proteinase k was added to the suspension.10 µL of lysozyme enzyme was added to the suspension and the tube was vortexes and incubated at 37 °C for 30 min. Then the tubes were incubated in a boiling water bath (100 °C) for 20 min, rapid cooling was done at -80 °C for 10 min. Then centrifugation at 10000 rpm for 5 min. was done. Then the supernatant which contained the DNA was transferred to a new Eppendorf tubes and stored at -20 °C until used for PCR amplification.

Polymerase chain reaction (PCR)
PCR Primers

The polymerase chain reaction (PCR) for amplification of *Staphylococcus spp.*16SrRNA gene was performed using iNtRON's Maxime PCR PreMix Kit (iNtRON i-Taq, South Korea) according to the manufacturer's instructions. (Intron Biogeotechnology Seongnam, Korea) Oligonucleotide primers used for amplification

of *Staphylococcus* 16SrRNA gene was described in Table 1. The steps of PCR amplification was performed, on ice, in mixtures of 25 µl reaction volumes containing 13 µl of distilled water, 2 µl of each of the forward and reverse primer, 5 µl of the extracted DNA and 5 µl of the master mix (Mgcl, Buffer, Taq Polymerase, DNTPs) (Intron Biogeotechnology Seongnam, Korea). The PCR thermocycling conditions were optimized, the samples were held initially for 5 min at 94 °C for initial denaturation, then followed by denaturation step at 94 °C for 45 seconds, primer annealing at 56 °C for 45 seconds, followed by the first step of elongation at 72 °C for 45 seconds and final elongation at 72 °C for 5 min.

Positive control obtained from previously sequences 16s gene and a negative control contains DW, primer and pcr mixture were used.

Agarose gel electrophoresis

A 1.5% agarose gel was prepared by dissolving 1.5 gm of agarose power into 100 ml of TBE buffer, then boiled in the microwave and cooled. 2 µl of Ethidium Bromide was added to the agarose gel, then the agarose gel was poured into gel tray caste inside the agarose gel electrophoresis apparatus and the wells was created by using a comb. The PCR products were poured into the agarose gel wells, the gel was covered by TBE buffer. The DNA in the agarose gel was separated by running at 120 V for 30 min.

Detection of the amplified PCR product

The products of The PCR were visualized with the UV trans illuminator then photographed. The size of *Staphylococcus spp.* 16S rRNA gene was determined using 100 bp molecular weight marker.

DNA Sequencing

DNA sequencing for *Staphylococcus* 16S rRNA gene (1200 bp) was performed for 6 samples which were positive by PCR. DNA purification in addition to standard DNA sequencing was performed in Macrogene Company (Seoul, Korea)

Sequence analysis and alignment

The obtained nucleotide sequences of *Staphylococcus spp.*16SrRNA gene (1200 bp) were matched for their sequence similarity with the respective genes using Nucleotide Blast (http// blast.ncbi.nlm.gov/Blast.cgi) [12].

Sequences were assembled using Codon Code program version (8.0.2).

Phylogenetic analysis

Phylogenetic trees for *Staphylococcus spp*., was constructed using MEGA 6 tree building program.

Data Management and analysis

Excel spread sheet was used for raw data entry. Then Log_{10} transformation of bacterial count was done, before the analysis, and SAS version 16.0 software was used for descriptive statistics. For all analysis, 95 % CI and P-value<0.05 was set for statistical significance of an estimate.

Results

The primary and secondary biochemical tests Table 2, showed the reaction of the *staphylococcus species* and *staphylococcus aureus* which was coagulate positive.

The study revealed that there are a significant different between the raw milk collected from farms and that vended by donkeys and cars were ($P \leq 0.05$) and the load of the bacteria (TVBC) was high in the three sources of milk Table 3.

Table 4 showed that (TVBC) had no significant deferent between the milk collected from farms in Khartoum and Khartoum North but there was significant different for that collected from Omdurman ($P \leq 0.05$) and there was no significant deferent of that vended by donkeys in the three areas but there was significant deferent of that vended by cars.

The percentage of contamination of raw milk by *Staphylococcus Spp.,* and *staphylococcus aureus* in Khartoum State were 72.2% Table 8 and 44.4% Table 12 respectively, while the percentage of contamination with *staphylococcus spp.,* and *staphylococcus aureus* in raw milk from farms was 68.3% Table 5 and 36.6% Table 9 respectively.

The percentage of contamination by *Staphylococcus Spp.,* and *staphylococcus aureus* of raw milk vended by donkey in Khartoum State were 75% Table 6 and 50% Table 10 respectively, while the percentage of contamination with *staphylococcus spp.,* and *staphylococcus aureus* of raw milk vended by cars were 73.3.3% Table7 and 46.3% Table 11 respectively.

TABLE 1. Oligonucleotide primers used for amplification of *Staphylococcus spp.* 16SrRNA gene

Target Gene	Primer Name	Primer sequence (5′→3′)	Size of PCR Product (bp)	Reference
16SrRNA	16SrRNA Forward	AGTTTGATCCTGGCTCCAG	1200	[11]
	16SrRNA Reverse	AGGCCCGGGAACGTATTCAC		

TABLE 2. Primary and Biochemical tests

No.	TEST	RESULT
1	Shape	Cocci in cluster shape
2	Gram stain	+ve
3	Growth Condition	Aerobic
4	Motility	None-Motile
5	Catalase	+ve
6	Oxidase	-ve
7	Oxidation- Fermentation	+ve
8	Deoxy ribonuclease (DNase) test	+ve for *staphylococcus aureus* -ve for other *staphylococcus spp.*
9	Coagulase test	+ve for *staphylococcus aureus* -ve for other *staphylococcus spp.*
10	Manitol	+ve for *staphylococcus aureus* -ve for other *staphylococcus spp.*

TABLE 3. Means of total viable count of bacteria [log_{10} cfu/ml] ±SD isolated from raw cows' milk in Khartoum State

Source	TVCB [log_{10} cfu/L] ±SD
Raw Cow´s milk from Dairy Farms	6.63 ± 0.07^{c}
Raw Cow´s milk vended by Donkey	6.90 ± 0.03^{a}
Raw Cow´s milk vended by cars	6.78 ± 0.12^{b}
P-value	0.0^{**}
$Lsd_{0.05}$	0.0533

Values are mean ±SD.
Mean value(s) having different superscript(s) are significantly different (P≤0.05)

TABLE 4. Means of Total Viable Count of Bacteria [log10 cfu/L] ±SD isolated from raw cows' milk from different areas in Khartoum State

Source	Omdurman	Khartoum	Khartoum North
Raw Cow´s milk from Dairy Farms	6.70 ± 0.09^{b}	6.60 ± 0.11^{c}	6.60 ± 0.11^{c}
Raw Cow´s milk vended by Donkey	6.91 ± 0.07^{a}	6.89 ± 0.10^{a}	6.90 ± 0.07^{a}
Raw Cow´s milk vended by cars	6.76 ± 0.13^{b}	6.78 ± 0.12^{a}	6.79 ± 0.09^{b}
P-value	0.0^{**}	0.0^{**}	0.0^{**}
$Lsd_{0.05}$	0.06584	0.04583	0.06877

Values are means.
Mean value(s) having different superscript(s) in a column are significantly different (P≤0.05)

TABLE 5. Numbers of *Staphylococcus Spp.* isolated from raw cow's milk in farms.

No.	Area	No. of samples	No. of Positive *Staphylococcus spp.*	Percentage of
1	Omdurman	20	12	20%
2	Khartoum	20	13	21.7%
3	Khartoum-North	20	16	26.6%
4	Total	60	41	68.3%

TABLE 6. Numbers of Isolated *Staphylococcus species* isolated from raw cow's milk vended by Donkey.

No.	Area	No. of Samples	No.. of Positive *Staphylococcus spp.*	Percentage
1	Omdurman	20	17	28.33%
2	Khartoum	20	14	23.33%
3	Khartoum-North	20	14	23.33%
4	Total	60	45	75%

TABLE 7. Numbers of *Staphylococcus species* isolated from raw cow's milk vended by car.

No.	Area	No. of Samples	No. of Positive *Staphylococcus spp.*	Percentage
1	Omdurman	20	15	25%
2	Khartoum	20	13	21.7%
3	Khartoum-North	20	16	26.6%
4	Total	60	44	73.3%

TABLE 8. Numbers of *Staphylococcus spp.* isolated from different sources of raw cow's milk in Khartoum State.

No.	Sources of milk	Numbers of Samples	Numbers of Positive *Staphylococcus spp.*	Percentage
1	Raw cow's milk in Farms	60	41	22.8 %
2	Raw cow's milk vended by Donkey	60	45	25%
3	Raw cow's milk vended by car	60	44	24.4%
4	Total	180	130	72.2%

Table 9. Numbers of *Staphylococcus aureus.* isolated from raw cow's milk in farms.

No.	Area	No. of Samples	No. of Positive *Staphylococcus aureus*	Percentage of
1	Omdurman	20	8	13.3%
2	Khartoum	20	6	10%
3	Khartoum-North	20	8	13.3%
4	Total	60	22	36.6%

Table 10. Numbers of isolated *Staphylococcus aureus* from raw cow's milk vended by Donkey.

No.	Area	No. of Samples	No. of Positive *Staphylococcus aureus*	Percentage
1	Omdurman	20	10	16.7%
2	Khartoum	20	9	15%
3	Khartoum-North	20	11	18.3%
4	Total	60	30	50%

Table 11. Numbers of *Staphylococcus aureus* isolated from raw cow's milk vended by car

No.	Area	No. of Samples	No. of Positive *Staphylococcus aureus*	Percentage
1	Omdurman	20	11	18.3%
2	Khartoum	20	8	13.3%
3	Khartoum-North	20	9	15%
4	Total	60	28	46.3%

TABLE 12. Numbers of *Staphylococcus aureus* isolated from different sources of raw cow's milk in Khartoum State.

No.	Sources of milk	No. of Samples	No. of Positive *Staphylococcus aureus*	Percentage
1	Raw cow's milk in Farms	60	22	12.2 %
2	Raw cow's milk vended by Donkey	60	30	16.6%
3	Raw cow's milk vended by car	60	28	15.5%
4	Total	180	80	44.4%

PCR and electrophoresis

Universal primers were used for amplification of 16S rRNA gene (1200 bp) of *staphylococcus spp*.. The PCR product was visualized on ethidium bromide-stained gel from DNA of the isolates (Fig. 1). Six samples of *staphylococcus spp.* were detected Using extracted DNA target , Negative control samples including samples without DNA target and nucleic acid-free water failed to demonstrate the 1200 bp.100bp molecular weight marker that used.

Sequencing and Phylogenetic Analysis

Sequencing of PCR amplicon and BLAST of 16s rRNA gene sequences:

Nucleotide sequences of 6 samples were determined for 16s rRNA gene, PCR products. Alignment of the *Staphylococcus spp.,* obtained sequences with reference strains from GenBank with sequence identity of 99% with strains from India, Italy, Turkey, China, Republic of Korea, USA, and Indonesia previously deposited in the GenBank (Table 13).

Phylogenetic analysis

The phylogenetic analysis conducted for the 16s rRNA gene of *Staphylococcus spp.,* showed a close relation of the 6 strain of *Staphylococcus spp.,* that isolated in this study with reference isolates previously identified Fig. 2.

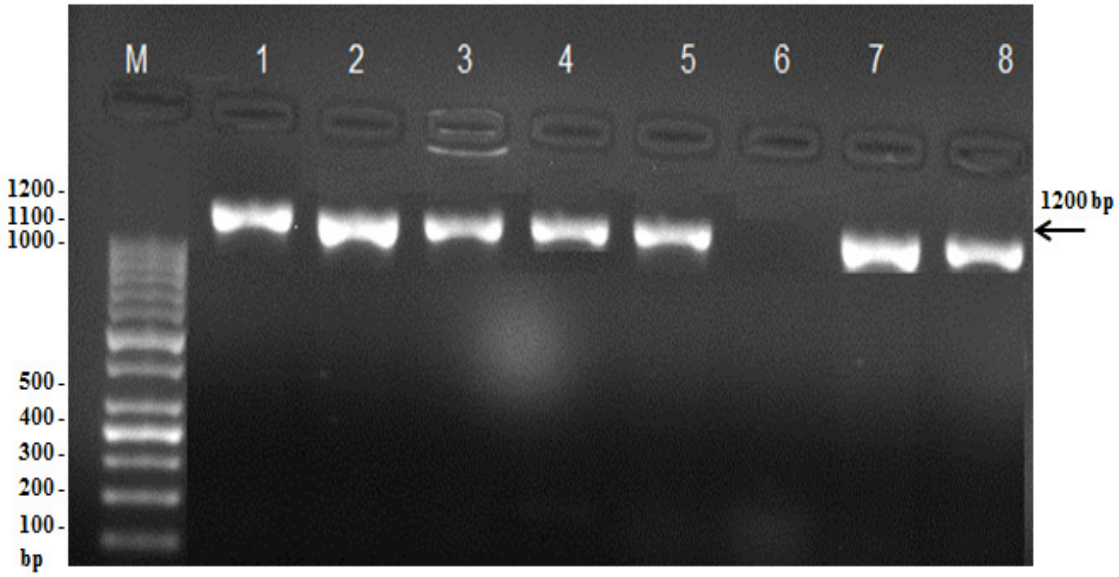

Fig. 1. show lane (M) marker (100bp) lane (1) positive control (1200 bp) lane (2), (3), (4), (5), (7) and (8) positive samples lane (6) negative control.

TABLE 13. Strain Names & Accession numbers of the Isolated *Staphylococcus spp.* and the identity % with some references in other countries.

[Sample Sequences (accession number)	Identity %	Organism	Location (accession number)
Seq1(MK010925)20	99%	*Staphylococcus. Epidemidis*	India (MH900188.1) Republic of Korea (MH144335.1). India (MH793436.1)
Seq2(KM010926)13	99%	*Staphylococcus. Epidemidis*	Italy (MK026828.1) China (MG755788.1) China (719590.1)
Seq3(KM010927)17	99%	*Staphylococcus. aureus*	Turkey (MH748546.1) China (MF511713,1) Pakistan (MH383087.1) Sir Lanka (MK000713.1)
١٥(Seq4(MK010928	99%	*Staphylococcus. aureus*	Indonesia (KX456107.1). India (KX821634.1) China (KC212028.1) India (KX821632)
Seq5 (MK010929)16	99%	*Staphlococcus.Smulans*	Turkey (KT372845.1) Turkey (KT372844.1) USA (KR822474.1)
Seq6(MK010930)12	99%	*Staphylococcus.Hominis*	China (MG254809.1) China (MG254783.1) India (MG255965.1)

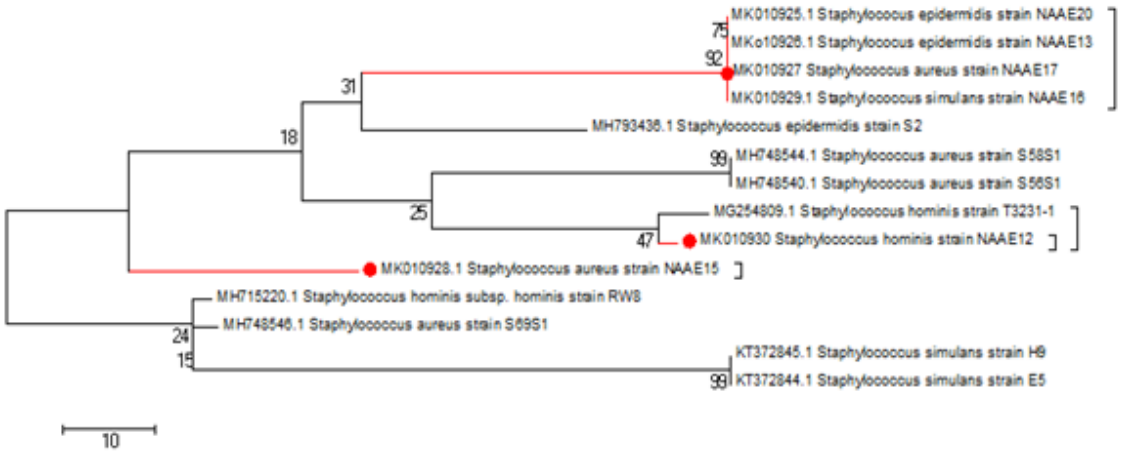

Fig. 2. Phytogenic Relationship of Isolated *Staphylococcus* (Red color) to different *Staphylococcus strain* references by neighbour-joining analysis of 16s rRNA gene ,based on genetic distances.

Discussion

The results of the total bacterial count in this study were presented in Table 3. All the raw milk had high bacterial load which were 6.63±0.07 $\log_{10}$ c.f.u./ml.,6.90±0.03 $\log_{10}$ c.f.u./ml., and 6.78±0.12 $\log_{10}$ c.f.u./ml., for raw milk from farm, raw milk vended by Donkey and raw milk vended by car respectively also there was a significant different between the three sources of milk. The results obtained were lower than that mention by [13] of raw milk from different sources in Shendi city who found that the higher count was in raw milk vended by donkey cart (8.09±1.11 Log_{10}), followed by milk from dairy farms (8.01±1.39 Log_{10}), milk vended in shops (7.99±1.21 Log_{10}) and pickup trucks (9.2±1.06 $_{\text{Log10}}$) also was lower than that mentioned by [14] who reported a significant ($P<0.001$) variation in total viable bacteria count from pickup trucks (Log_{10} 9.22±0.84), farms (Log_{10} 9.06±0.64) and vended on donkey cart (Log_{10} 8.82±0.84) in Khartoum state. The TVBC of raw cow's milk obtained from the current results (Table 3) are also lower than that found by [15] in 6 districts in Ethiopia where the $\log_{10}$ cfu/ml were 7.36 ± o.17 to 7.88 ± 0.13. In another study in three different districts in Ethiopia [16] they found that the total aerobic bacterial counts (TABC) of raw milk obtained at farmer level has an average value of 6.88±0.46 $\log_{10}$ cfu/ml., which was within the rang in this study, the results were also higher than that found by [17] In India they found that all the samples of raw milk had high bacterial load with average ranged from 4.19+0.69 to 6.35+0.11 $\log_{10}$ c.f.u./ml. [18] reported that the percentage of isolated *Staphylococcus aureus* was from raw cow's milk in Khartoum state, was 42.2% from farm and 53.1% from vendors, which is higher than that found in this study for cow's milk from farm was 36.6%, vended by Donkey 50% and vended by cars 46.3% this may be due to improvement of veterinary services and training, also it lower than the result of other study in Ethiopia [19] reported that the prevalence of *staphylococcus aureus* was significantly greater in raw milk samples (47%), in other study in Ethiopia also it was 42.9%, [20] in different other studies were in Morocco, [21] S. aureus was found to be 40%. In Pakistan [22] found that 36.9% of raw milk was contaminated by *staphylococcus aureus* and in India [23] found that 61% of raw milk which are higher than the result found in this study. In the other hand there were researches done and found to have lower prevalence than this study like 18.18% prevalence of *S. aureus* found in Turkey by [24] the results in thesis studies are lower than this study might be due to the better prevention and hygienic environment than study area of this research.

Bacterial count was high due to milking dirty udders, maintaining an unclean milking and housing environment and failing to rapidly cool

milk to less than 4°C., and lack of extension and training among the labourers.

Conclusion

In conclusion, it was found that most of the raw milk sold in Khartoum State is of low hygienic quality, so the authorities which responsible of dairy management should enforce all the regulations needed for producing and purchasing raw milk with acceptable hygienic quality.

Consumption of raw milk may also pose health hazards as milk is highly prone to microbial growth and may harbours pathogens. Staphylococcus aureus and other Staphylococcus spp. may impose public health hazard in dairy products. Therefore, further studies must be done on the enterotoxigenic potential of the isolates,

Pasteurization of raw milk must be used as it is widely adopted and most effective method to ensure completely destruction of all pathogenic and spoilage microorganisms commonly found in milk and inactivation or reduction of other non-pathogenic spoilage bacteria.

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Conflict of interest

The authors declare no conflict of interest.

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عزل والتعرف الجزيئي لانواع المكورات العنقودية في حليب البقر المنتشرة بولاية الخرطوم

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هدفت هذه الدراسة إلى التعرف وتقييم الحمل البكتيرى ومستوى التلوث بأنواع المكورات العنقودية والمكورات العنقودية الذهبية في حليب البقر الخام الموزع بولاية الخرطوم. تم أخذ مائة وثمانين عينة من الخرطوم وأم درمان وشمال الخرطوم ، و 60 عينة من الحليب الخام من أبقار في المزارع تبدو صحية ، و 60 عينة من الحليب الخام تحملها الحمير ، و 60 عينة من الحليب الخام التي تباع فى السيارات. تم فحص عدد البكتيريا الكلى باستخدام حساب الطبق القياسية. تم فحص العينات لوجود أنواع المكورات العنقودية باستخدام الطرق التقليدية، المزروعة في Baird-Parker و Mannitol agar ،صبغة جرام ، ثم باستخدام الاختبارات البيوكيميائية.

أظهر TVBC أن أعلى حمل بكتيري تم اكتشافه في الحليب الخام الذي تم بيعه بواسطة نقله بالحمير (log10 cfu / ml 03. ± 6.90) تم يلية المباع عن طريق السيارات (log10 cfu / ml 0.12 ± 6.78) ثم أدنى حمولة بكتيرية تم اكتشافها في الحليب الذي تم جمعه من مزرعة (log10 cfu / ml 0.07 ± 6.63). من بين 180 عينة من الحليب الخام المفحوصة ، أظهرت 130 تلوثًا من أنواع المكورات العنقودية المقابلة لـ 72.2 ٪ من العينات الملوثة ومن أصل 180 عينة من حليب البقر الخام 80 ملوثة بالمكورات العنقودية الذهبية المقابلة لـ 44.4 ٪ من العينات. تم تأكيد أنواع المكورات العنقودية المعزولة أكثر باستخدام تفاعل البلمرة المتسلسل (PCR) الذي يستهدف التسلسل الجزئي لجينات 16 rRNA. أظهرت النتائج مستوى عال من التلوث بالمكورات العنقودية. والمكورات العنقودية الذهبية في حليب البقر الخام التي تتوزع بولاية الخرطوم.

الكلمات الدالة: لبن البقر ، الممرض ، المكورات العنقودية ، التلوث.