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Isolation and Molecular Identification of *Escherichia Coli* from cow's milk in Khartoum State

Nadia A. Elshiekh[1,2], Galal E. Mohammed[3], Mohammed A. Abdalla[3], Hisham Altayeb[4], Osama M.Elkhair[5]

1 Department of Pathology, Parasitology and Microbiology, College of Veterinary Medicine, Sudan University of Science and Technology, P.O. Box 204 Kuku, Khartoum-North, Sudan
2 Department of Dairy Sector, Ministry of Animal Resources , P.O. Box 293 Khartoum, Sudan.
3 Department of Preventive Medicine, College of Veterinary Medicine, Sudan University of Science and Technology, P.O. Box 204 Kuku, Khartoum-North, Sudan
4 Department of Microbiology-Molecular biology, Collage of Medical Laboratory Science, Sudan University, P.O. Box
5 Department of Microbiology, Central Laboratory, Ministry of High Education Scientific Research
Corresponding Author: Nadia A. Elshiekh

The aim of this study was to evaluate the total load of bacteria, and detection of Escherichia coli contamination in raw, heated and pasteurized cow´s milk consumed in Khartoum state, and to characterize the isolated microorganisms using PCR and DNA sequences technique.
A total of 300 samples were taken from Omdurman, Khartoum and Khartoum North, for which 60 samples of raw milk from farms, 60 samples of raw milk vendor by donkeys, 60 samples of raw milk vendor by cars 60 samples of milk heated and sold in groceries and 60 samples of pasteurized milk sold in groceries all milk samples transported to laboratory at 4°C . The Total Viable Bacterial count was done for all (1.98±0.05 log10 cfu/ml). The organisms which isolated by conventional methods were out of 180 samples of studied raw milk, 112 showed contamination by Escherichia coli, corresponding to 62.2% of the samples being contaminated. In heated milk out of 60 samples 12 samples were contaminated by Escherichia coli, corresponding to 20%. The pasteurized milk out of 60 samples, 3 samples were contaminated with this microorganism corresponding to 5% of the samples being contaminated.
Identification of the isolated Escherichia coli. confirmed further for molecular characterization, by using the Polymerase Chain Reaction (PCR), and partial sequences of 16s rRNA gene.
The results obtained in this study showed a high level of bacteriological quality and contamination by E.coli of milk distributed in 3 localities by different channels in Khartoum state and it was directly affect the human health and can cause a public illness and also affect dairy industry.

***Key words**: Raw milk, Pasteurized milk, heated milk Pathogenic microorganism, PCR, 16s rRNA gene, Escherichia coli, Contamination.*

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I. Introduction

Milk is an ideal environment for microbial growth due to its enriched ingredients, and for this reason 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.

Bacterial contamination of raw milk can originated from different sources such as air, milking equipment, feed, soil, faeces and grass. The number and type of microorganism in milk immediately often after milking are affected by a different factors such as animals and equipment clean less, season feed and animal health. The pathogenic bacteria which may found in milk can results with high health risk and may be the main reasons of consumer's illness. In Sudan, milk is produced mostly in non-standardized way and is usually supplied to the consumers of the urban and rural areas by milkmen and by some established dairy farms where surplus milk is readily available. Milk vendor on Donkeys, cars, and sold in groceries. Contaminated raw milk can be a source of pathogenic bacteria. Different heat and other treatments are given to raw milk in order to remove pathogenic microorganism and to increase the shelf life as pasteurization is a process applied to some food and food products in order to get red of the microbiological risk and to increase their storage period. Microbial load and incidence of the bacterial pathogens in foods are indicators of food quality (Rosmini et al., 2004), as well as the sanitary conditions of its production (Guerreiro et al., 2005). Poor microbiological quality of raw milk due to bacterial contamination (Ahmed and Abdellatif, 2013), inadequate packaging system (Singh

et al., 2012) and improper temperature control (Malek, et al., 2012), favour microbial growth and metabolism and lead to undesirable changes thereby shorten the shelf life of milk (Fromm and Boor, 2004).Microbiological analysis of pasteurized milk indicated presence of pathogens like *E. coli* and *S. aureus* (Fadhedi et al., 2013), (Woldemariam and Asres 2017) .

Escherichia coli is a Gram-negative, nonsporulating, short rod bacilli shaped, flagellated, and facultative anaerobic bacteria belonging to the Enterobacteriaceae Family (Bavaria, 2012).

The presence of *E. coli* in milk is an indicator of faecal contamination and can survive for brief period outside the body make them an ideal indicator organism to test environmental samples for faecal contamination so the objectives of this study is to identify the isolated *E.coli* from milk samples using sensitive and specific tool of identification PCR and molecular sequences technique also to evaluate the level of contamination of milk distributed in Khartoum state from different milk sources.

II. Material and Methods

A total of 300 samples were collected randomly in sterile containers from Omdurman, Khartoum and Khartoum North, for which 60 samples of raw milk from farms, 60 samples of raw milk vended by donkeys, 60 samples of raw milk vendor by cars 60 samples of milk heated in groceries and 60 samples of pasteurized milk sold in groceries . All samples were 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:

Serial were done from10^{-1} to 10^{-8} then from the diluent 10^{-4} and the diluent 10^{-5} 1 ml was taken and cultured on nutrient agar medium then were incubated for 24 hours at 37° C then the total viable bacterial count (TVBC) were made, (Houghtby, et al 1992).

Isolation and identification of *E. coli* bacteria were done by using conventional methods, cultured in MacConkey and EMB agar, then the cultured were examined with naked eyes for growth and colony morphology. gram Stain, primary and secondary test also were done.

Then the confirmation of isolated *Escherichia coli* was done by molecular technique using polymerase chain reaction (PCR) at research laboratory, Sudan University of Science and Technology.

Confirmation of isolated Escherichia coli by molecular Technique

DNA Extraction:

50 µL of distilled water was transferred to Eppendorf tube, pure colonies (n.3) of isolated *Escherichia coli* transferred to the Eppendorf tube, 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 centrifuged at 10000 rpm for 5 min. Then the supernatant which contained the DNA was transferred to new Eppendorf tubes and stored at -20°C until used for PCR amplification.

The concentration of the extracted DNA was measured by spectrophotometer at 260/280 wave length.

Polymerase chain reaction (PCR)

PCR Primers:

The polymerase chain reaction (PCR) for amplification of *Escherichia coli* 16S rRNA gene was performed using iNtRON's Maxime PCR PreMix Kit (iNtRON Biotechnology, Taq, Seongnam, South Korea) according to the manufacturer's instructions. Oligonucleotide primers used for amplification of *Escherichia coli* 16S rRNA gene as described in Table 2. 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 forward and reverse primers (1 µl of each one) , 5 µl of the extracted DNA and 5 µl of the master mix (Mgcl, Buffer, Taq Polymerase and DNTPs) (Intron Biogeotechnology Seongnam, Korea). The PCR thermocycling conditions were optimized, the samples were held initially for 3 min at 94°C for initial denaturation, then followed by denaturation step at 94°C for 30 seconds, primer annealing at 56°C for 30 seconds, followed by the first step of elongation at 72°C for 30 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.

Table 1. Oligonucleotide primers used for amplification of Escherichia coli 16S rRNA gen

Target Gene	Primer Name	Primer sequence (5´→3´)	Size of PCR Product (bp)	Reference
16SrRNA	16SrRNA Forward 16SrRNA Reverse	AGTTTGATCCTGGCTCCAG AGGCCCGGGAACGTATTCAC	528	Tsen et al. (1998)

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 (Tris Boric EDTA) buffer. The DNA in the agarose gel was separated by running at 120 V for 30 min.

Detection of the amplified PCR product:

The PCR products were visualized with the UV trans- illuminator and photographed. The size of *Escherichia coli* 16SrRNA gene was determined using 100 bp molecular weight marker.

Sequencing of PCR products and BLAST of 16s rRNA gene:

DNA sequencing for *Escherichia coli* 16S rRNA gene (528 bp) was performed for 14 samples which were positive by PCR. DNA purified and sequenced in Microgen Scientific Services Company (Seoul, Korea).
BLAST of16s rRNA gene sequences:

Sequence analysis and alignment:

The obtained nucleotide sequences of *Escherichia coli* 16S rRNA gene (528 bp) were matched for their sequence similarity with the respective genes using Basic Local Alignment Search Tool (Blast) of the GeneBank (http//blast.ncbi.nlm. nih.gov/Blast.cgi) (Atschul et al., 1997).
Sequences were assembled using Codon Code program version (8.0.2).
Sequences were screened for chimera by using the submitter bio-edit version 7.2.5.

Nucleotide sequences submission to GeneBank:

Sequences submission to the GeneBank was conducted following the instructions via the web tool Bankit of GeneBank http://www.ncbi.nlm.nih.gov/WebSub .

Phylogenetic analysis:

Phylogenetic trees for *Escherichia coli* strains were constructed using MEGA 6 software (Tamura et al., 2013). The DNA sequences were analyzed by manual alignment editing and submission to the MEGA 6 tree-building program.

Data Management and Analysis

Microsoft excel spread sheet was employed for raw data entry. Then Log_{10} transformation of bacterial count was done and statistical analysis was performed using Statistical Analysis Systems (SAS version 9.2 2016) software was used for descriptive statistics. For all analysis, 95 % CI and P-value<0.05 was set for statistical significance of an estimate.

III. Results and Discussion

The results were summarized in the following tables:

Table2: Biochemical Reaction of E.coli

NO	Test	Result
1	Lactose fermentation	+ve
2	Citrate Utilization	-ve
3	Indole production	+ve
4	Urease test	-ve
5	Kliglers Iron Agar(KIA) test	+ve yellow colour with gas production no H2s
6	Voges-proskauer(V-P) test	-ve
7	Methyl Red test	+ve

Table (3): Means of total viable count of bacteria [log_{10} cfu/L] ±SD of cows' milk in Khartoum State

Source	TVCB [log_{10} cfu/L] ±SD
A	6.63 ± 0.07^{c}
B	6.90 ± 0.03^{a}
C	6.78 ± 0.12^{b}
D	4.62 ± 0.08^{d}
E	1.98 ± 0.05^{e}
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 of cows' milk from different areas in Khartoum State

Source	Omdurman	Khartoum	Khartoum North
A	6.70 ± 0.09^{b}	6.60 ± 0.11^{c}	6.60 ± 0.11^{c}
B	6.91 ± 0.07^{a}	6.89 ± 0.10^{a}	6.90 ± 0.07^{a}
C	6.76 ± 0.13^{b}	6.78 ± 0.12^{a}	6.79 ± 0.09^{b}
D	4.63 ± 0.11^{c}	4.52 ± 0.40^{c}	4.72 ± 0.15^{d}
E	1.99 ± 0.11^{d}	1.98 ± 0.11^{d}	1.98 ± 0.11^{e}
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).

Key:
A ≡ Dairy farm
B ≡ Milk vender on donkey
C ≡ Milk vendor by car
D ≡ Boiled milk vendor in groceries
E ≡ Pasteurized milk sailed in groceries

Table 5: Numbers of *E.coli* isolated from different Sources of cow's milk in Khartoum State

No.	Sources of milk	Numbers of Samples	Numbers Positive *E.coli*	Percentage
1	Raw cow's milk in Farms	60	34	11.3%
2	Raw cow's milk vendor by Donkey	60	40	13.3%
3	Raw cow's milk vendor by car	60	38	12.7%
4	heated cow's milk in groceries	60	12	4%
5	Pasteurized cow's milk from groceries	60	3	1.0%
6	Total	300	127	42.3%

The results obtained in this study (table 4) showed that there were significant differences in the total bacterial count of milk from different sources, the higher count was in milk from venders on donkey (Log_{10} 6.90±0.03), followed by milk venders on car (Log_{10} 6.78±0.12) then milk from dairy farms (Log_{10} 6.63±0,07). respectively. These results are lower than that mentioned by Rahamtalla *et al*. (2016) 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 venders on donkey cart (Log_{10} 8.82±0.84) in Khartoum state. It was also lower than that found by Ibtesam (2017) who mentioned that there was no significant difference in the total bacterial count of milk from different sources in shendy although the higher count was in milk from venders on donkey cart (Log_{10} 8.09±1.11), followed by milk from dairy farms (Log_{10} 8.01±1.39), milk vending shops (Log_{10} 7.99±1.21) and pickup trucks ($_{Log10\ 7}$.92±1.06)

The log_{10} cfu/ml of TVBC of raw cow's milk obtained from the current results were also lower than that found by (Worku et al., 2012) 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 3 different districts in Ethiopia (Amistu, et al., 2015) 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.

The results showed that all the raw milk had high bacterial load which ranged from log_{10} cfu/ml 6.63±0.07 to 6.90±0.03. The most frequent cause of high bacterial load is poor hygienic practices of the milking system as dirty udders, maintaining an unclean milking and housing environment and lack of cooling facilities to rapidly cool of milk to less than 4°C so bacterial contamination of raw milk indicates substandard hygienic conditions practiced during production and subsequent handling and distribution which include poor hygienic and control measures applied to the farms, similar results were reported by (Kas *et al*., 2013).

The TVBC detected in the pasteurized milk in this study was (log10 cfu/ml 1.98±0.05) which was lower than that found by (Nato, etal 2016) who reported that the quality of pasteurized market milk in Kenya has a mean of Total Viable Count (TVBC) Log_{10} cfu/ml 5.64±.19 which were above the EAS requirement as it is of Log_{10} cfu/ml 4.4.8 cfu/ml.

In this study the percentage of *E.coli* contamination in raw milk was 42.3% (table4) which was lower than that reported by (Amistu, et al.,2015) who isolated 26 out of 60 (43.33%) from different critical points site of milk chain and was higher than that reported by (Olfa et al., 2013) 13 out of 50 milk samples (26%) also higher in Sfax in Tunisia (32.5%) were contaminated with *E. coli* from raw cow's milk from different localities, and this may due to unhygienic milking practise, water supplied used for washing the utensils, handling, time, and also high temperature could be the sources for accelerating the number of bacterial contamination of milk .

(Salman and Hamad, 2011) isolated and identified different species of coliforms from vender and market milk of Khartoum State, Sudan which included *E. coli* with the percentage of (32%). The significant difference between sailed milk in farmers and that vendor by donkey or cars also reported by (Orregard 2013) as he reported a significant difference between farmers and agents, and between farmers and shop's milk, while no significant difference was observed between small–scale and large–scale agents' milk in terms of total bacterial counts.

(Fadaei, 2014) studied 300 samples of raw milk sample with poor hygienic practices in dairy environment he found that 125 samples were contaminated with *Escherichia coli* (41.66%).

In this study the Log_{10} c.f.u./ml of the pasteurised milk was 1.98±11 to 1.99±11 which was lower than that found by (Saxena and Rai, 2013) in pasteurized milk samples as they ranged from Log_{10} c.f.u. 3.43±0.17 to Log_{10} c.f.u./ml 8.2±0.05. The percentage of *E.coli* that found in pasteurised milk in this study were 3 out of 60 (5.0%) which was lower than that found by (Fadhedi, et al., 2013) which was (9%) in 4 different areas in Iran, also microbiological analysis of pasteurized milk done by (Singh et al., 2011) showed the present of *Escherichia coli*. In Bangladesh (Saha and Ara, 2012) reported that the standard plate count (SPC) of the pasteurized milk samples ranged from 54200 to 68400 cfu/ml, which is much higher than that recommended by Bangladesh standard (must not exceeding 20,000 cfu/ml).The high bacterial count in the pasteurized milks may be due to defective pasteurization machinery, and or post-pasteurized contamination due to poor hygienic practices by workers. In Albania a study of pasteurized milk by Dini (2012), showed that the contamination by *E. coli* was 1.6% which was higher than that found in this study.

The milk which heated in groceries and sailed in different opened containers showed high level of bacterial load (Log_{10} c.f.u. 4.62±0.08) and contamination of *Escherichia coli* were they 12 out of 60 samples (20%) this may due to post contamination from the environment, containers, handling and storage .

PCR and electrophoresis

Universal primers were used for amplification of 16S rRNA gene (528 bp) of Escherichia *coli*. The PCR product was visualized on ethidium bromide-stained gel from DNA of *E. coli* (Fig. 1). 14 strains of *E.coli* were detected Using extracted *E. coli* DNA target, Negative control samples including samples without DNA target and nucleic acid-free water failed to demonstrate the 528 bp.100 bp molecular weight marker that used.

Sequencing and Phylogenetic Analysis:

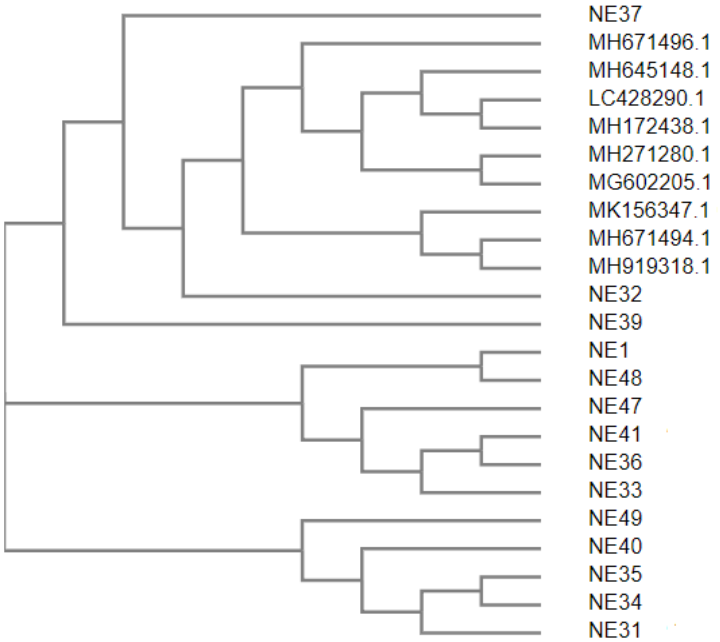
Sequencing of PCR amplicon and BLAST of 16s rRNA gene sequences:

Nucleotide sequences of 14 samples were determined for 16s rRNA gene, PCR products. Alignment of the *Escherichia coli* obtained sequences with reference strains from GenBank showed that the isolates belong to *Escherichia coli* with 99% sequence identity with *Escherichia coli* strains from USA, South Korea, India, China, Brazil previously deposited in the GenBank (Table 4).

Phylogenetic analysis

The phylogenetic analysis conducted for the 16s rRNA gene of *Escherichia coli* showed a close relation of the *Escherichia coli* isolated in this study with reference isolates previously identified in the Figure below:

Figure 1: Phytogenic Relationship of Isolated *Escherichia Coli* to different *E.coli* references.



Nucleotide sequences:
Sequences were submitted to the GeneBank database followed the instructions in the web tool Bankit of the NCBI GeneBank database, http://www.ncbi.nlm.nih.gov/WebSub/?tool=genebank. 14 Nucleotides sequences in this study were deposited in the NCBI GeneBank database under accession numbers: MH978997.1 – MH979010.

Table 6: Strain Names & Accession numbers of the Isolated *E.coli* and the identity % with some references in other countries:

Strain Name & accession no.	% Identity	Organism	Location & accession no.
NE1(MH978997.1)	99.27%	*E.coli*	China (MK621228.1)
NE31(MH979008.1)	99.63%	*E.coli*	China (MK156347.1)
			China (MH919318.1)
NE32(MH978998.1)	99.63%	*E.coli*	India (MH1724381)
NE33(MH978999.1)	99.63%	*E.coli*	Iraq (Lc428290.1)
NE34(MH979000.1)	99.63%	*E.coli*	Nigeria (MK606100.1)
NE35(MH979001.1)	99.63%	*E.coli*	China (MK 621232.1)
			China (MK621211.1)
NE36(MH97002.1)	99.63%	*E.coli*	India (MH271280.1)
NE37(MH97003.1)	99.80%	*E.coli*	China (MG602205.1)
NE39(MH97004.1)	99.81%	*E.coli*	China (MK621241.1)
			China (MK621221.1)
NE40(MH97005.1)	99.44%	*E.coli*	Nigeria (MK332563.1)
NE41(MH97006.1)	99.44%	*E.coli*	India (MH271278.1)
NE47(MH97007.1)	99.62%	*E.coli*	India (MH645148.1)
NE48(MH97009.1)	99.63%	*E.coli*	Saudi Arabia (MG798649.1)
NE49(MH97010.1)	99.63%	*E.coli*	India (MH486679.1)

In this study PCR confirmed 90% positive (14) of the convenial positive samples the remaining samples may be considered as false positive results may be due to insufficient DNA or inhibitor factors.

The PCR is the most Sensitive method than the convenial method but the PCR facilities are not always available especially in developing countries.

The results of the analysis of the sequences, alignment and phylogenetic tree showed a close relation among 14 isolates.

IV. Conclusion &Recommendation

The result obtained in this study concluded that raw milk available to the consumer in Khartoum state via different suppliers chain have a high bacterial load beyond acceptable critical limits according to Sudanese Standards and European community member states. Also the milk considered from the study areas were

contaminated with most hazardous agents such as *E .coli*,. It indicates that good hygienic, production and farming practise was not strictly followed during milk production, handling, storage and distribution.

Consumption of raw milk and direct consumption of heated milk in groceries may pose health hazards as milk is highly prone to microbial growth and may harbours pathogens.

Pasteurization must be done perfectly as it is widely adopted as a 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.

The results of molecular study have identified *Escherichia coli* in cow´s milk which is very important as indicator of milk contamination with pathogenic strain.

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