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Microbiological Evaluation and Molecular Discrimination of Milk Samples from Humans and Different Animals

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ABSTRACT

Milk is a highly nutritious food and it is important to be free of any pathogenic microbes that could be transmitted to humans and affect public health. A total of 145 milk samples were collected from humans and different animal species (cow, buffalo, ewe, goat, camel, mare, and donkey) and underwent physical examination (color, odor, and taste), chemical analysis for its components (water, total salt, fat, protein, lactose, and ash), and finally microbiological (bacteriological and mycological) examinations. Standard plate count, preliminary incubation count, lab pasteurized count, coliform, *Escherichia coli*, *Staphylococcus* species, *Salmonella* species, yeast, and mold counts were measured. PCR test was performed to differentiate milk from different sources (animals and humans) by producing a specific band for each milk type. The results of the physical examination of different kinds of milk showed different grades of white color with the characteristic odor and taste of each milk type. Chemical examination revealed that the highest water content was in donkey milk and the lowest was in buffalo milk while total solids indicated the highest content in sheep milk and the lowest in donkey milk. The microbiological analysis presented that the standard plate count results were the highest in the milk obtained from sheep and camel, while donkey milk was the least in this regard. Yeast counts were the highest in buffalo milk but cow milk was the highest in mold counts. PCR results of milk types using species-specific primers and DNA template extracted from milk somatic cells revealed a specific band for each milk type as 157, 195, 225, 242, 274, and 711 base pair (bp) for goat, human, sheep, buffalo, cattle milk, and camel milk, respectively. It was concluded that more restrictions must be applied to decrease milk contamination as high microbial counts detected in the present study can affect milk quality, public health, and the dairy industry. PCR used in the current work for milk discrimination used milk somatic cells specifically mitochondrial cytochrome b gene which exhibited high specificity in the PCR reactions and this could be served as a cheap and simple method, compared to other types of PCR.

Keywords: Bacterial count, Fungal count, Milk, PCR

INTRODUCTION

Milk serves the nutritional and physiological needs of the offspring. It is high-quality nourishment and a nearly complete human food that can be consumed without any processing steps (Mehta, 2015; Roy et al., 2020). Milk microbial constituents (bacteria and fungi) vary and originate from different sources of defilement, such as the udder skin, milking utensils cleanliness, water, air, animal feed, grass, housing circumstances, fecal matter, and soil (Quigley et al., 2013; Machado et al., 2017).

Pathogenic bacteria present in milk is often considered as a major public health concern, especially for immunocompromised individuals. Keeping fresh milk at a high temperature together with unhygienic practices during the milking process may also result in low-quality milk (Chatterjee et al., 2006). Many milk-borne diseases are transferable to humans through raw or unpasteurized milk consumption (Parekh and Subhash, 2008).

Bacterial counts are determined in raw milk before processing, and their results express the health condition of the mammary gland. Hygiene demands for animals in production herds prevent milk collection from ill animals. Non-hygienic circumstances create serious hazards for customers' health because microbiologically infected raw milk constitutes a source of pathogenic microbes and milk-borne illnesses for humans. The existence of milk-borne diseases is more common in the population consuming raw milk than in those consuming pasteurized milk (Pyz-Łukasik et al., 2015). Raw milk is mainly considered as a perfect growth medium for microbes, including many fungal elements, as raw milk contains all essential nourishments and circumstances that support their growth (Gulbe and Valdovska, 2014). Several studies assured that yeasts and molds can contaminate milk from various sources as ambient air and farmworkers. In most cases, yeasts are the most common, but in some ecosystems, molds are predominant, indicating that the relative proportions of yeasts and molds can differ significantly (Lavoie et al., 2012). Many different methods have been used for the identification of species, including chromatographic (Pellegrino et al., 1991), immunological (Addeon et al., 1995), electrophoretic (Cartoni et al., 1998), reversed-phase high-performance liquid chromatography, and ELISA (Haasnoot et al., 2014), as well as chemical methods (Makadiya and Pandey, 2015).

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Recently, molecular approaches have been used for species discrimination, and it has been recommended due to its simplicity, sensitivity, repeatability, and reproducibility (Bottero et al., 2003). PCR method has been successfully used to identify different meats from domesticated animals and meat products (Abd El-Razik et al., 2019; Abuelnaga et al., 2021). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay is one of the recent molecular techniques which can be applied for the differentiation of various types of milk. Besides, PCR-RFLP has a lower cost in comparison with other methods, such as real-time PCR (Abdel-Rahman, 2017; Abd El-Razik et al., 2019). The cytochrome b gene was reported to be highly polymorphic and could be used to differentiate the buffalo from cow species (Bellagamba et al., 2001).

The aim of the present study was to investigate the microbial diversity of raw milk from human and different animal species. In this regard, PCR was used to make a differentiation between milk species from humans and those of different animal species. The result of the present study could have an important impact on improving the quality of the raw milk and dairy product industry.

MATERIALS AND METHODS

Ethical approval

The current work was approved by the Medical Research Ethics Committee, National Research Centre, Egypt (19156).

Sampling

A total of 145 milk samples, including 5 milk samples from humans (according to guidelines described by Lovelady et al., 2002) and 20 samples from each animal species from Baladi breeds (cow, buffalo, sheep, goat, camel, donkey, and horse) were collected from June 2020 to June 2021. Milk samples were examined physically (color, odor, and taste), chemically, and microbiologically and the results were recorded.

Chemical analysis of milk samples

Milk samples collected from humans and different animal species (cow, buffalo, camel, ewe, goat, mare, and donkey) underwent chemical analysis according to Mehta (2015).

Microbiological evaluation of milk

Bacterial counts of microorganisms in milk

The examined milk samples were subjected to standard plate count, preliminary incubation count (psychrotrophs), Lab pasteurized count (in a water bath at 77.6°C for 30 seconds), and coliform count at the NRC laboratory, Egypt, according to the methods described by Martin et al. (2011).

Detection of microorganisms in milk

Detection of different microbial contaminants present in milk was conducted as previously performed by Quinn et al. (2011).

Escherichia coli count

Escherichia coli (*E. coli*) was identified and confirmed by colony morphology on eosin methylene blue agar (EMB) (Oxoid company) and performing biochemical tests according to Bergey and Holt (1994).

Staphylococcus count

According to the plate count technique of APHA (1992), *Staphylococcus aureus* was counted using the direct plate count method on Baird Parker agar enriched with egg yolk tellurite emulsion (Oxoid company) (Lancette and Bennett, 2001).

Salmonella count

Isolation and quantification of *Salmonella* were carried out using the method described by Quinn et al. (2002). The samples were enriched by inoculating a sterile swab from milk sample into 5 ml of Rappaport Vassiliadis broth (Sigma-Aldrich, Inc., USA) and incubated at 37ºC for 24 hours. Then, a loopful of enriched Rappaport-Vassiliadis broth was streaked onto xylose lysine desoxycholate (XLD) agar (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37ºC for 24 hours. The colonies were examined for the characteristic red colonies with the black center of *Salmonella* with or without hydrogen sulfide. In the next step, several biochemical tests were performed following the standard protocol (Cappuccino and Sherman, 1996).

Fungal count in milk samples

The milk samples were serially (10^2 to 10^6) diluted in sterile 0.1% (w/v) peptone solution, then tenfold serial dilutions were performed for counting of fungi under complete aseptic conditions (Lavoie et al., 2012). In the next step, 1 ml of the prepared milk dilutions was added into a petri dish in duplicate. Then, 10-20 ml molten sabouraud dextrose agar (SDA, cooled to 42-45°C) were poured into each petri dish. The media and the dilutions were blended by whirling

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gently clockwise and anti-clockwise and were left until solidification at room temperature (Soliman et al., 2019). Cultured plates were put upside down to prevent contamination and they were kept in an incubator at 25ºC for 3-5 days. Yeast colonies that were defined by being creamy, dull-white, pink, yellow, regular, and irregular patterns were counted utilizing a colony counter and yeast count/gram was estimated and recorded. Moreover, for mold count, the plates were kept at 25ºC for 5-7 days at a reversed position. Amid the incubation time, the plates were inspected routinely for the characteristic star-shaped mold structure, and colonies were numbered and indexed (APHA, 1992).

Polymerase chain reaction

DNA extraction

Milk samples (25 ml) from each milk type were gathered from cattle, buffalo, sheep, goat, camel, horse, donkey, and human, and centrifuged at 2200 g for 5 minutes for sedimentation of milk specimen. Then, 1 ml of the sediment was re-mixed with 200 µl TE (1 mM EDTA, 10 mM Tris-HCl (pH = 7.6), and 300 µl 0.5 M EDTA, pH = 8), and centrifuged at 3000 g for 10 minutes to prevent blocking by casein (Murphy et al., 2002; Psifidi et al., 2010). Milk pellet was then diluted in 200 µl of phosphate-buffered saline and DNA was extracted using GF-1 Tissue DNA extraction kit (Cat.-No.GF-TD-050, Vivantis Co., Malaysia) according to the company instructions with elution of DNA in 50 µL of elution buffer, then DNA was stored at -20°C until use.

Polymerase chain reaction

The reaction was applied in 25 µl reaction volume containing 12.5 µl of 2 × COSMO PCR RED Master Mix (Cat. W1020300X, Willofort Co., UK.), 1 µl (0.1 mM) of each primer, 9.5 µl of Double distilled water, and 1 µl of the purified DNA. The reaction steps composed of one cycle of 95°C for 2 minutes taken after 35 cycles of 95°C for 1 minute, annealing for 30 seconds (Table 1), 72°C for 45 seconds, and the final extension at 72°C for 10 minutes (GS-96 gradient thermocycler, Hercuvan, Malaysia). The amplification PCR products were visualized by 1.5% agarose gel electrophoresis colored with ViSafe Red Gel Stain, Vivantis Co., Malaysia). PCR products and 100 bp DNA ladder were electrophoresed at 100 V and examined using InGenius3 gel documentation system (Syngene, UK).

Statistical analysis

Results were measured statistically for descriptive analysis (mean, maximum, minimum, and standard error) utilizing SPSS 14.

Table 1. Species-specific PCR primers for the amplification of human, cattle, buffalo, sheep, goat, camel, horse, and donkey milk samples in Egypt during 2020-2021

Species	Sequence 5'- 3'	Annealing temperature	PCR product	Reference
Cattle	(Forward) GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA (Reverse) CTAGAAAAGTGTAAGACCCGTAATATAAG	60°C	274bp	Matsunaga et al. (1999)
Buffalo	(Forward) TAGGCATCTGCCTAATTCTG (Reverse) ACTCCGA TGTTTCATGTTT CT	61°C	242bp	Rajapaksha et al.(2003)
Sheep	(Forward) TTAAAGACTGAGAGCATGATA (Reverse) ATGAAAGAGGCAAATAGATTTTCG	58°C	225bp	Ilhak and Arslan (2007)
goat	(Forward) GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA (Reverse) CTCGACAAATGTGAGTTACAGAGGGA	58°C	157bp	Matsunaga et al. (1999)
Camel	(Forward) ACCACATTTCAACTATTTCAAAACCG (Reverse) ATGTACGGCTGCGAGGGCGGTAA	61°C	711bp	Deng et al. (2020)
Horse	(Forward) CTATCCGACACACCCAGAAGTAAAG (Reverse) GATGCTGGGAAATATGATGATCAGA	61°C	153bp	Kesmen et al. (2010)
Donkey	(Forward) ATCCTACTAACTATAGCCGTGCTA (Reverse) CAGTGTTGGGTTGTACACTAAGATG	57°C	145bp	Kesmen et al. (2007)
Human	(Forward) CAGCAGCCATTCAAGCAATGC (Reverse) ATCGGTGGGTTAAGTTTATTAAGTGT	60°C	195bp	Kapoor et al. (2013)

RESULTS AND DISCUSSION

Milk is a great microbial growth medium when the temperature is optimum. It is easily contaminated and spoiled in case it is processed in an unsanitary manner. Many milk-borne outbreaks of human illnesses have spread due to the contamination of milk by unclean dairy employees' hands, unclean utensils, flies, and contaminated water supplies (Oliver et al., 2005).

Physical examinations of milk specimens were performed in the current study and the results indicated different grades of white color with the specific odor and taste for each type of milk (Table 2). The chemical analysis presented the average composition of different milk specimens (Table 3). In the present work, the milk samples contained water content of 88, 83, 80.6, 86, 87.8, 87.4, 90.9, and 89% in cow, buffalo, sheep, goat, camel, human, donkey, and horse milk, respectively. However, Mehta (2015) obtained 84.2, 86.3, 83.7, 86.5, 87.61, and 87.43% in buffalo, cow, sheep,

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goat, camel, and human milk, respectively. Total solids in the current study were 12.35, 16.5, 17.5, 12.8, 12.1, 12.5, 11.5, and 13.8% in cow, buffalo, sheep, goat, camel, human, donkey, and horse milk, respectively, but Park and Haenlein (2006) and Guha et al. (2021) showed 14.4, 12.1, 16.3, 10.2 and 11% in camel, goat, sheep, donkey, and mare milk, respectively.

These differences in constituents of different types of milk components may be due to nutritional, genetic, and environmental variables that influence both major and minor components. The composition of milk also influences its suitability as a raw material for various dairy products, as well as its nutritional value and organoleptic and physicochemical properties (Alichanidis et al., 2016). According to the pasteurized milk ordinance standard, the maximum bacterial count in raw milk is 10^5 cells per ml. Several studies have also shown a high total bacterial count in milk samples. The high count may be due to milk handling and contamination from animal bedding and these results match with the current study in standard plate count results. The present work revealed that goat milk was the highest in standard plate count while camel milk was the least (Hayes et al., 2001; Muhammad et al., 2009; Lingathurai and Vellathurai, 2010). Minj and Behera (2012) observed that in cow's milk the average total viable count of rural milk specimens was 8.257 ± 0.937 log CFU/ml and that of the urban milk specimens was 8.756 ± 0.803 log CFU/ml. Nearly similar counts were also obtained concerning the preliminary incubation counts of rural and urban milk specimens. The mean counts of preliminary incubation values in rural specimens were 8.522 ± 0.929 log CFU/ml and the urban specimens were 8.889 ± 0.424 log CFU/ml. Moreover, for lab pasteurized count (LPC), the average bacterial load of the rural specimen was 8.083 ± 0.081 log CFU/ml and that of the urban milk specimen was 7.500 ± 0.739 log CFU/ml. Lower results were reported by Massouras et al. (2020) as they reported that the total aerobic mesophilic and psychrotrophic counts ranged 2.18-2.71 log CFU/ml and 1.48-2.37 log CFU/ml, respectively.

The bacterial counts in different types of milk are presented in Table (4). The preliminary incubation count provides a more accurate picture of psychrophilic (cold-loving) bacteria and the quality of cleanliness on the farm. Before making any conclusions, the preliminary incubation count should always be compared to the Total viable count of the fresh and un-incubated samples. According to the American Public Health Association (APHA, 1992), the highest allowed preliminary incubation count is 200000 CFU/ml, however, counts as low as 50000 CFU/ml are still possible. The preliminary incubation count in the current work is considered within the permissible limit. The highest preliminary incubation count was in goat milk and the least was in ewe milk.

The LPC is frequently used to assess the efficacy of farm hygienic conditions providing the relative number of organisms that may survive in the pasteurized milk specimen. Lab pasteurized counts in warmed milk were significantly lower than standard plate counts. Counts more than 300 CFU/ml are indicative of a source of contamination. Elevated LPC values are typically associated with chronic or repeated cleaning problems; the bacteria isolated from the LPC can survive pasteurization, but most of them cannot reproduce in refrigeration temperature and remain static, and some even vanish (Murphy and Carey, 2007). The results showed that the highest lab count was in goat milk while the least was in camel milk. Moreover, a high bacterial count was in goat milk and it was indicative of high contamination.

The absence of most pathogenic bacteria in camel milk might be due to the activity of protective proteins (Lysozyme, Lactoferrin, Lactoperoxidase, Immunoglobulin G and A) of camel milk. As reported by Barbour et al. (1984) and El-Agamy (1992), camel milk lysozyme (LZ) was effective against *Salmonella*. Lactoperoxidase was bacteriostatic against the Gram-positive strains and showed a bactericidal effect against Gram-negative cultures. Coliforms are considered typical flora of human and animal digestive tracts and several milk-borne outbreaks of human illness have been propagated. They have been employed as bacteriological quality indicators for milk and its products (Chatterjee et al., 2006). In the present work, the coliform count showed the highest value in donkey milk samples $1.1 \times 10^3 \pm 2.4 \times 10^2$ and the least load was in camel milk $8.9 \times 10 \pm 2.2 \times 10$, while the highest *E. coli* value was observed in buffalo milk $8.5 \times 10 \pm 0.5 \times 10$ and the least was in cow milk $2 \times 10 \pm 0.4 \times 10$. *Staphylococcus* (*Staph*) species showed the highest count in goat milk $4.6 \times 10^3 \pm 1.7 \times 10^3$ and the least count in sheep milk as $2.36 \times 10^2 \pm 0.66 \times 10^2$. Minj and Behera (2012) recorded in cow's milk higher values of the enteric count, while Adugna and Eshetu (2021) showed a lower count in the coliform count.

Table 2. Physical examination of different milk samples from animals and humans in Egypt during 2020-2021

Species	Color	Odor	Taste
Human	Little white	Normal	Sweet
Cow	Yellowish white	Characteristic fresh	Sweet
Buffalo	Creamy white	Characteristic fresh	Sweet bitter
Sheep	Bright white	Freshly milk sheepy flavor	Creamy sweet
Goat	Very white	Characteristic odor of freshly milk goat	Viscous sweet
Camel	Very white	Freshly milk camel	Salty due to vitamin C
Donkey	White	Good palatable	Very sweet due to lactose
Horse	White	Good palatable	Very sweet due to lactose

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Table 3. Chemical analysis of different milk samples from animals and humans in Egypt during 2020-2021

Milk	Water (%)	Total solids (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)
Human	87.4	12.5	1.8	1.9	7.1	0.2
Cow	88	12.35	3.8	3.7	4.6	0.7
Buffalo	83	16.5	6.8	4.2	4.9	0.8
Sheep	80.6	17.5	7.4	5.5	4.7	0.9
Goat	86	12.8	4.4	3.9	4.1	0.8
Camel	87.8	12.1	3.0	2.5	1.3	0.7
Donkey	90.9	11.5	1.4	2.0	6.9	0.4
Horse	89	13.8	1.9	2.5	6.8	0.3

Table 4. The bacterial count in different types of milk in Egypt during 2020-2021

		Cow	Buffalo	Camel	Sheep	Goat	Horse	Donkey	Human
Standard plate count	Minimum	2×10^3	1×10^4	2×10^4	1×10^4	1×10^4	8×10^2	8×10^2	1×10^2
	Maximum	8×10^6	6×10^6	4×10^6	6×10^6	6×10^7	1×10^7	6×10^6	3×10^3
	Mean±SE	$1.6\times10^6 \pm 0.52 \times 10^6$	$1.17 \times 10^6 \pm 0.39 \times 10^6$	$8.9 \times 10^5 \pm 2.7 \times 10^5$	$8.9 \times 10^5 \pm 3.3 \times 10^5$	$8.6 \times 10^6 \pm 3.2 \times 10^6$	$1.3 \times 10^6 \pm 0.57 \times 10^6$	$1.1 \times 10^6 \pm 0.41 \times 10^6$	$1.3 \times 10^2 \pm 5.6 \times 10^2$
Preliminary incubation	Minimum	1×10^2	1×10^2	1×10^2	1×10^2	1×10^2	1×10^2	100	6×10
	Maximum	3×10^5	8×10^4	8×10^4	2×10^4	2×10^6	4×10^4	8×10^4	1.1×10^2
	Mean±SE	$2.5\times10^4 \pm 1.4\times10^4$	$2.3 \times 10^4 \pm 6.6 \times 10^3$	$7.9 \times 10^3 \pm 4.09 \times 10^3$	$2.7 \times 10^3 \pm 1.02 \times 10^3$	$1.02\times10^5\pm9.9\times10^4$	$3.8 \times10^3 \pm 1.9 \times 10^3$	$5.5 \times 10^3 \pm 3.9 \times 10^3$	$9.4 \times 10 \pm 0.9 \times 10$
Lab pasteurized	Minimum	100	100	100	100	100	100	100	0
	Maximum	4×10^3	1×10^3	3×10^2	3×10^3	2×10^4	8×10^2	3×10^3	0
	Mean±SE	$2.9 \times 10^2 \pm 2 \times 10^2$	$1.3 \times 10^2 \pm 0.5 \times 10^2$	$0.4 \times 10^2 \pm 0.18 \times 10^2$	$2.9 \times 10^2 \pm 1.49 \times 10^2$	$1.7 \times 10^3 \pm 1.08 \times 10^3$	$0.8 \times 10^2 \pm 0.4 \times 10^2$	$1.9\times10^2 \pm 1.4 \times 10^2$	0
Coliform	Minimum	3	90	95	10	9	10	100	0
	Maximum	1.1×10^3	7×10^3	4×10^2	1×10^3	9×10^4	1×10^3	5×10^3	5×10
	Mean±SE	$9.7 \times 10 \pm 5.3 \times 10$	$9.29 \times 10^2 \pm 3.8 \times 10^2$	$8.9 \times 10 \pm 2.2 \times 10$	$1.3 \times 10^2 \pm 0.52 \times 10^2$	$4.9 \times 10^3 \pm 4.4 \times 10^3$	$1.8 \times 10^2 \pm 0.6 \times 10^2$	$1.1 \times 10^3 \pm 2.4 \times 10^2$	10 ± 10
Escherichia coli	Minimum	2	20	40	12	9	20	50	0
	Maximum	9.3×10	100	1×10^2	100	100	2×10^2	1×10^2	0
	Mean±SE	$2 \times 10 \pm 0.4 \times 10$	$8.5 \times 10 \pm 0.5 \times 10$	$2.7 \times 10 \pm 0.8 \times 10$	$3.7 \times 10 \pm 0.9 \times 10$	$6.9 \times 10 \pm 0.8 \times 10$	$5.9 \times 10 \pm 1.1 \times 10$	$5.3 \times 10 \pm 0.9 \times 10$	0
Staphylococcus species	Minimum	1×10^2	1×10^2	8×10	9	1×10^2	100	100	0
	Maximum	1×10^4	9×10^3	1×10^3	1×10^3	3×10^4	3×10^3	9×10^3	10
	Mean±SE	$1.7\times10^3 \pm 5.8\times10^2$	$2.7 \times 10^3 \pm 7.8 \times 10^2$	$2.5 \times 10^2 \pm 0.66 \times 10^2$	$2.36 \times 10^2 \pm 0.66 \times 10^2$	$4.6 \times 10^3 \pm 1.7 \times 10^3$	$5.05 \times 10^2 \pm 1.7 \times 10^2$	$1.3 \times 10^3 \pm 5.4 \times 10^2$	$0.2 \times 10 \pm 0.2 \times 10$

Mean ± SE: Mean ± standard error

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In Buffalo milk, similar results in the coliform count were obtained by Gurler et al. (2013) as they recorded mean values of 2.95 ± 0.21 log10 cfu/ml and nearly equal counts in *Staph* species count (2.46 ± 024 log10 cfu/ml) and lower count in *E. coli* (1.10 ± 0.17 log10 cfu/ml). However, Han et al. (2007) recorded lower coliform and *Staph* species count (2.42 and 1.68 log10 cfu/ml), respectively, and a higher count of *E. coli* (1.53 log10 cfu/ml). In sheep and goat milk, Ombarak and Elbagory (2017) recorded lower counts of coliform and *Staph* species with mean count values of 6.47 ± 2.17×10^5 and $1.66 \pm 0.85 \times 10^5$ CFU/ml in goat and ewe milk samples, respectively, and *Staph* species with mean count values of 1.41×10^4 and 6.67×10^4 CFU/ml in goat and ewe milk, respectively. In camel, Bassuony et al. (2014) recorded no detection of *E. coli, Staph*, and *Salmonella* and obtained a lower coliform count.

In mares, Czyzak-Runowska et al. (2018) did not detect the pathogenic *Salmonella* spp. and coliforms in the raw milk but Bauzad et al. (2019) reported a high rate of *E. coli* contamination in buffalo milk samples in all udder milk and farm milk samples in Curio and that may be due to several factors. These factors were mainly the low hygiene and sanitation during raw milk production at the farm level. According to Nurwantoro and Mulyani (2003), *E. coli* contamination could be caused by poor handling of milk, inadequate sanitation, and the environmental factors of the mesophilic temperature and the neutral pH.

Elevated count values of yeasts and molds in milk are quite uncommon because of the neutral pH of milk bacteria to prevail and their existence in large counts in milk is considered unacceptable due to its ability to deteriorate the sensory evaluation of milk (Lues et al., 2003).

In the present study, yeast and mold counts of different types of milk showed the highest yeast count in goat milk and the least in donkey milk, while the highest mold count was in cow milk and the least in mare milk (Table 5).

Higher fungal counts of 3.71 ± 0.83 cfu/ml were obtained by Adugna and Eshetu (2021) from milk samples in Ethiopia. In buffalo milk, Gurler et al. (2013) in Turkey recorded lower counts (2.63 ± 0.25 log10 cfu/ml), compared to the current study. Han et al. (2007) recorded a higher fungal count in buffalo milk as they reported 1.79 log10 cfu/ml in China. Regarding camel milk, Bassuony et al. (2014) recorded no detection of yeast in Egypt while Ismaili et al. (2016) detected high yeast and mold count in Egypt as the counts of yeast and mold were 3.13×10^6 and 1.60×10^5 cfu ml, respectively.

In the present work, *Salmonella* was not isolated. Ombarak and Elbagory (2015) obtained the same result as they recorded that *Salmonella* was not isolated in any of examined raw milk samples. Bogdanovičová et al. (2016) did not detect *Salmonella* in sheep and goat milk while Abbas et al. (2013) recorded *Salmonella* in a low percentage (6.67%). The presence of *Salmonella* and other types of pathogenic bacteria in milk has been the cause of public health problems, especially for those persons who consume *Salmonella* contaminated milk.

Previous studies have assured that breast milk contains an important variety of bacteria that can be transmitted to the babies together with various other nourishments and immunological components. These bacteria are beneficial and could have a protective effect. They can also stimulate the immune system and add some of the first colonizers in the infant microbiome. Boix-Amorós et al. (2017) obtained higher bacterial and fungal counts as they reported 8.9×10^5 cells/ml and 3.5×10^5 cells/ml for bacterial and fungal counts, respectively.

Many approaches, such as chemical, immunological, and molecular approaches, have been used to determine the species origin of raw milk. PCR-RFLP (Abdelfatah et al., 2015), multiplex PCR (Bottero et al., 2003), Real-time PCR (Liao et al., 2017), and DNA-based fluorometric approach (Kounelli et al., 2017) are some of the molecular methods available for distinguishing closely related species.

Polymerase chain reaction used in the present study was ordinary PCR which could be cost-effectively and simply applied, compared to other types of PCR. For the identification of cattle, buffaloes, sheep, goat, camel, horse, donkey, and human milk samples, the accuracy of the species-specific primers and ideal PCR circumstances were utilized.

For molecular identification of milk from humans and different animals (buffaloes, sheep, goat, camel, horse, donkey, and human milk), species-specific primers and ideal PCR circumstances were performed. The primers yielded distinct species-specific PCR products of 274, 242, 225, 157, 711, and 195 bp for cattle, buffaloes, sheep, goat, camel, and human milk samples, respectively (Figure 1). Horse and donkey milk DNA provided the expected products (153 bp and 145 bp, respectively) as shown in Figures 2 and 3. These PCR bands were produced exclusively from the DNA retrieved from animal and human mitochondrial cytochrome b genes and exhibited no cross-matching with the DNA from other species Figures 1-3.

The present work implemented a fast, sensitive, practical, animal-friendly, and cost-effective source of genomic DNA extraction from milk somatic cells of different animals (cattle, sheep, goats, and horses). Milk was considered an excellent source of genomic DNA, and 10 ml of raw milk was sufficient to yield a significant volume of DNA appropriate for molecular analysis, such as PCR (Psifidi et al., 2010; Pokorska et al., 2016). Six different DNA extraction techniques were optimized, tested, and compared for the extraction of DNA from ovine milk samples. The primers used in the present study showed high specificity in the PCR reaction and succeed in the discrimination of different milk samples obtained from humans and animals.

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Table 5. Total yeast and mold count of different types of milk in Egypt during 2020-2021

	Yeast			Mold		
	Minimum	**Maximum**	**Mean±SE**	**Minimum**	**Maximum**	**Mean± SE**
Cow	20	1×10^3	$2.4 \times 10^2 \pm 7.6 \times 10$	8	4×10^2	$4 \times 10 \pm 1.9 \times 10$
Buffalo	10	4×10^2	$7.5 \times 10 \pm 2.2 \times 10$	6	9×10	$2.3 \times 10 \pm 0.7 \times 10$
Sheep	15	1×10^2	$7.4 \times 10 \pm 1.1 \times 10$	8	7×10	$3.4 \times 10 \pm 0.7 \times 10$
Goat	14	3×10^3	$6.19 \times 10^2 \pm 2.3 \times 10^2$	7	6×10	$2.1 \times 10 \pm 0.5 \times 10$
Horse	9	1×10^2	$1.8 \times 10 \pm 0.8 \times 10$	5	2×10	$0.3 \times 10 \pm 0.1 \times 10$
Camel	10	1×10^2	$2.7 \times 10 \pm 0.8 \times 10$	3	6×10	$0.4 \times 10 \pm 0.3 \times 10$
Donkey	12	9×10	$0.6 \times 10 \pm 0.4 \times 10$	10	1×10^2	$1.2 \times 10 \pm 0.6 \times 10$
Human	5	1.5×10	$0.56 \times 10 \pm 0.2 \times 10$	1	0.3×10	$0.13 \times 10 \pm 0.058 \times 10$

Mean ± SE: Mean ± standard error

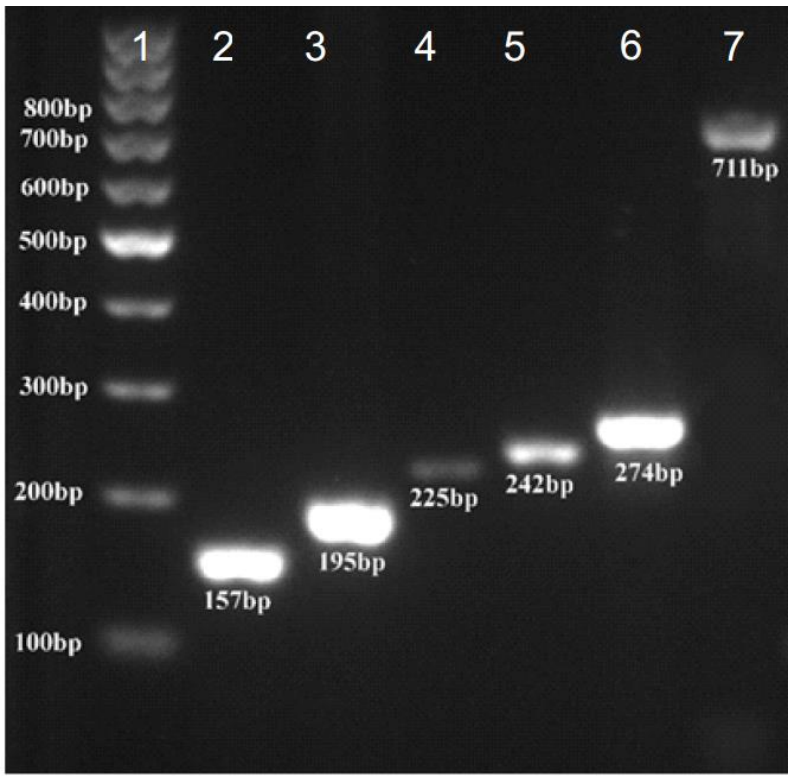

Figure 1. The PCR product of different types of milk amplified with species-specific primers. 1: Molecular marker (100 bp), 2: Goat milk,; 3: Human milk, 4: Sheep meat, 5: Buffalo milk, 6: Cattle milk, 7: Camel milk

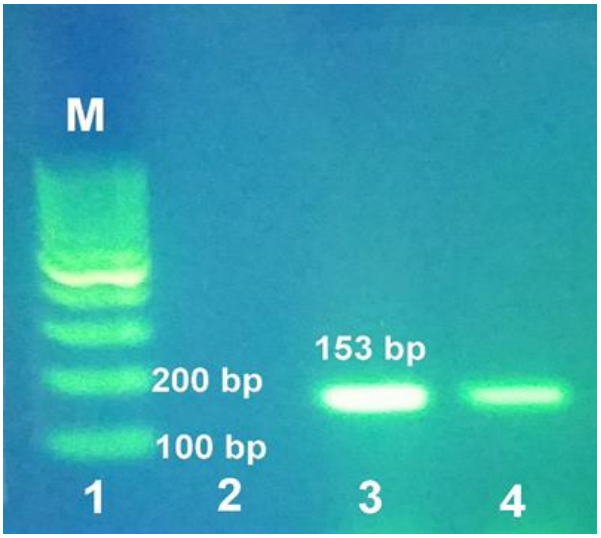

Figure 2. The PCR product amplified with horse-specific primers. 1: Molecular marker (100 bp), 2: Negative control, 3 and 4: Horse milk samples (153bp)

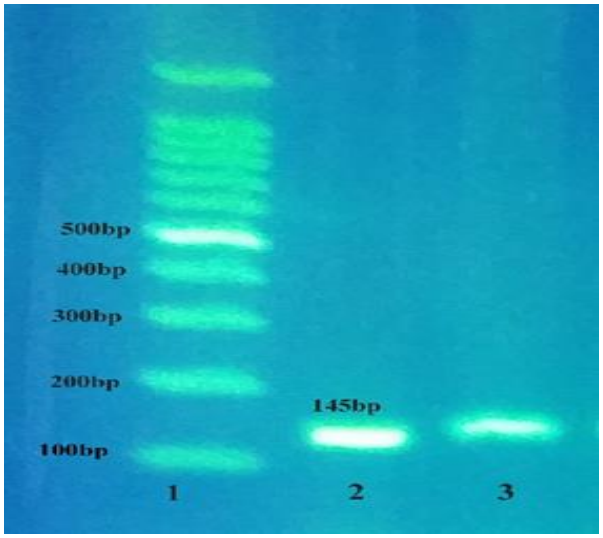

Figure 3. The PCR product amplified with donkey-specific primers. 1: Molecular marker (100 bp), 2: Negative control, 3 and 4: Donkey milk samples (145bp).

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CONCLUSION

Milk is considered as a complete food for human beings as it is rich in various constituents that can also support the growth of different microbes, so monitoring microbial contamination of milk implemented in the current work is crucial to protect human beings from milk-borne microbes. In the present study, DNA was extracted from milk somatic cells specifically mitochondrial cytochrome b gene which exhibited high specificity in the PCR reactions. This method can succeed in the identification of eight different types of milk. Therefore, it can serve as a simple, sensitive, and reproducible method to be easily applied and those results will be the core of further studies on milk and its byproducts.

DECLARATIONS

Authors' contribution

Azza Sayed Mohammed Abuelnaga and Nagwa Sayed Ata designed the study, participated in performing the experiments and analyzing the data. Khaled Abd El-Hamid Abd El-Razik participated in designing the study, performing the experiments, and writing the manuscript. Riham Hassan Hedia participated in analyzing the data and writing the manuscript. Mona Mohamed Hassan Soliman participated in performing the experiments and writing the manuscript. Mai Mohamed Kandil, Elgabry Abd-Elalim ELgabry, and Amany Ahmed Arafa participated in performing the experiments and analyzing the data. All authors checked and confirm the final draft of the manuscript before submission to the journal.

Competing interests

The authors declare that they have no competing interests.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

Consent to publish

The authors agreed to publish the article.

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