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The Milk Borne Pathogens of Raw Milk from Some Egyptian Farms in Different Seasons

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Authors' contributions

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ABSTRACT

Milk has good quality protein and is a unique substance in that it is consumed as fluid milk with minimal processing and also it is the raw material used to manufacture a wide variety of products. Milk is susceptible to contamination by many pathogenic microorganisms, which result in infection and threat to consumer's health. The aim of this study was to determinate occurrence of pathogenic microorganisms in raw milk in four seasons from different locations in Egypt, the obtained counts results showed that the samples gave the lowest Total Plate Count (TPC) of $3x10^5$cfu/ml in winter's samples. While, the summer's sample showed the highest TPC of $5.8x10^7$cfu/ml. *E. coli* count ranged from $2x10^2$cfu/ml to $5.8x\ 10^5$cfu/ml which the lowest count was noticed in winter's samples. Staphylococcal count ranged from $2.7\ x\ 10^3$cfu/ml (winter sample) to$1.28\ x\ 10^6$cfu/ml (another sample in the same season). These results indicated poor hygienic standard of raw milk from uncontrolled environments and the increased public health risk of those consuming raw milk from such uncontrolled sources and all these tests consume time but with Culture-independent methods that are based on protocols where total DNA (or RNA) is directly extracted from the substrate it can save time. Coupled with a global analysis, these methods make it possible to study the total diversity from the bulk extract in a single step.

Keywords: Milk Pathogens; raw milk bacteria; TTGE method.

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1. INTRODUCTION

Milk is a complex biological fluid containing about 100,000 different molecular species in several states of dispersion. Milk provides an ideal nutrition source for microorganisms and generally has a pH value in the range needed to contribute to proliferation. The undesirable microorganisms in milk can be classified into two major groups' spoilage microorganisms and pathogenic microorganisms. Coliforms and other gram negative bacteria, which can be associated with unsanitary production; thermoduric bacteria, which can survive pasteurization conditions; spore formers, which produce the heat- and desiccation-resistant structures known as spores; pathogens that cause mastitis, which can be shed into the milk by infected udders; and various yeasts and molds [1,2]. A variety of microbes with human pathogenic potential, including *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Mycoplasma bovis* and *Corynebacterium bovis* can sometimes be found in raw milk [1,2,4]

The rate of microbial contamination of raw milk is influenced by the health status and hygiene of dairy cows, hygiene of the environment in which dairy cows are housed and milked, methods of udder preparation and milking techniques, methods used for the cleaning and disinfection of milking machines and milk tanks, hygiene of the attendant staff [5].The number and types of micro-organisms in milk after milking immediately are affected by factors such as animal and equipment cleanliness, season, feed and animal health [6].Cold storage of milk on farms minimizes the growth of mesophilic microflora but it has brought about a new problem because low temperatures allow the growth of psychrotrophic microflora in milk [7].Thus, the microbiological content of raw milk affects quality, shelf life, and safety of processed milk and other dairy products.

The knowledge about bacterial diversity is useful for understanding the nature of the sample to be studied. Studies of the microflora and its relation to ecosystems have traditionally focused on the cultivable fraction of the bacteria present. Detection of pathogenic bacteria in milk involves, species identification in milk can be accessed through using of either culture dependent or culture-independent methods; selective enrichment subculture on selective agar plates followed by confirmatory identification tests according to either morphological, biochemical or genetic characteristics (culture dependent methods). These methods include colony isolation, phenotypic characterization (including morphology, and biochemical tests), and this can take several days to complete. The weaknesses of phenotypic methods comprise poor reproducibility and discriminatory power, laboriously investigations, and the ambiguity of some techniques caused by complex growth conditions [8,9]. Moreover, species occurring in low numbers are often out-competed *In Vitro* by numerically more abundant microbial species [8] and some species may be unable to grow *In Vitro*. However, culture-dependent methods are time-consuming, due to long culture periods and elaborate culture techniques. Hence, if culture conditions are poor and the number of isolates too low, the culture collection will not be representative of the community and the actual microbial diversity will be misinterpreted. Different cultures can even be bulked and analyzed using global analysis methods, such as culture-independent methods [10]. These methods have already shed light on the structure of microbial populations in milk [11].

Community-level studies are relying more and more on culture-independent methods that based on the direct analysis of DNA without any culturing step. As they are fast and potentially more exhaustive, these methods are well suited for analyzing microbial communities over time and may provide the possibility of exploring cheese microflora dynamics in detail. Most of these methods use polymerase chain reaction (PCR) amplification of total DNA [10].

Applications of culture independent molecular methods are needed to improve our understanding of the total microbiota, as the conventional culture-based methods are selective and do not cover the entire microbial diversity of complex environments [9]. More rapid molecular methods have been developed to analyze diversity within bacterial communities. These methods are based on direct analysis of DNA in the environment and do not require cell cultivation. They include single-stranded conformational polymorphism analysis, denaturing gradient gel electrophoresis (DGGE), and temporal temperature gradient gel electrophoresis (TTGE) as mentioned by Delbès et al., [12], Randazzo et al., [13], Duthoit et al., [14] and Ogier et al., [15]. All of these approaches involve extraction of nucleic acids (DNA or RNA), amplification of genes encoding 16S rRNA, and analysis of PCR products by a genetic fingerprinting technique [9].

In Egypt, about 80% of produced milk is collected from a lactating animal (cow and buffalo) twice a day and is recognized as a highly microbial contamination due to milk-handling practices from milking a few animals by hand in the out of doors. In countries with developing economies, it is not uncommon to find small quantities of non-refrigerated milk being hauled by individual producers to collection centers for entry into the market or use without heat treatment in cheese making. So, the objective of this study was to detection of possible origin spoilage & pathogenic microorganisms with analyzing the microbial content in raw milk in the four seasons using traditional and modern methods.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Twenty one raw cow's milk samples (250 ml) were collected from several areas of Alexandria and Behira in Egypt. Samples were collected at four different times of the year corresponding to the four seasons. All the samples were collected in wide mouth sterile (100 ml) containers aseptically, labeled and immediately transported to the laboratory in an ice-box where they were processed immediately.

2.2 Standard Plate Count (SPC)

Numerous selective and differential tests used to determine the specific types of bacteria in raw milk. Serial dilutions of raw milk were used for microbial enumeration in quarter strength Ringer's solution. Appropriate dilutions prepared in Ringer's solution and plated on different types of selective media for total count bacteria using plate count agar (PCA, biolife. Italy), coliform and *Enterobacter* sp. count using Violet Red Bile Glucose Agar (VRA, biolife. Italy), *Staphylococcusaureus* count using Vogel and Johnson Agar (VJA. biolife. Italy), thermophilic spore forming bacilli using Nutrient Agar, Enter *Coccus* spp. using *Streptococcus faecalis* Medium (SF. biolife. Italy) and *Salmonella* spp. using Xylose lysine deoxycholate agar (XLDA. biolife. Italy). All the plates were incubated at 37ºC for 48 hours, except the plates for the determination of thermophilic spore forming bacilli were incubated at 45ºC for 48 hours; cultures were further examined by Gram staining.

2.3 Culture Independent Method

2.3.1 Total DNA extraction

Thirty ml of each all tested raw milk samples were dissolved in 20 ml of sterile trisodium citrate 2% and homogenized by using Ultra-turrax until the solution was opaque. To each

sample, 50 mg Pronase (SIGMA-ALDRICH) and 100µl of β-mercaptoethanol were added. This was followed by incubation at 50˚C for 3 hours. Both milk fat and proteins were removed by sample centrifugation at 12.000 rpm\ 15min (Sigma, 2-16PK, Germany).Then the pellet of bacteria was washed twice with 1ml TES buffer. Whole DNA was extracted using Wizard DNA purification Kit as described with the manufacturer (promega, Madison, Wiscon. USA). Whole DNA was analyzed by 0.8% agarose gel (Amersham Biosciences, Sweden) electrophoresis.

2.3.2 Reference strains setup

We selected two bacterial species from bacteria with low-G-C-content genomes belonging to the genera Enterococcus sp., and Staphylococcus sp. to ensure the reproducibility and significance of patterns obtained by TTGE. Three strains were generally selected from each species group.

2.3.3 Nested -PCR amplification

The V3 region of the 16S rRNA gene is the substrate for PCR amplification. The extracted DNA (1 µl) was amplified by two successive PCR amplification 700bp fragment, including the 16s rRNA gene region was the first PCR amplification by using the outer primers W01 (5'-AGA GTT TGA TC (AC) TGG CTC-3') and W012 (5'- TAC GCA TTT CAC C (GT) C TAC A-3'). The reaction mixture (25 µl) consists of 50 ng of bacterial DNA, 60 pmol of each primer, puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Sweden) which included deoxynucleoside triphosphate at a concentration of 200 PM, 2.5 U of puRe Taq DNA polymerase, 10 mMTris-HCl, (pH 9.0), 50 mMKCl, 1.5 mM MgCl2 and enough sterile deionized water to bring the volume to 25 µl.

Then the PCR fragment containing V3 region was used as substrate to amplify an approximately ~200bp fragment by using the inner primers HDA1-GC-Clamp (5'- CGC CCGGGGCGC GCC CCG GGC GGG GCGGGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3') (–GC-clamp is underlined) and HDA2(5'-GTA TTA CCG CGG CTG CTG GCA-3'). The reaction mixture (25µl) consists of 50 ng of bacterial DNA, 60 pmol of each primer, 1µ Dimethyl sulfoxide (DMSO), Ready-To-Go PCR Beads (Amersham Biosciences, Sweden) which included deoxynucleoside triphosphate at a concentration of 200 PM, 2.5 U of puReTaq DNA polymerase, 10mMTris-HCl, (pH 9.0), 50mMKCl, 1.5 mM MgCl2 and enough sterile deionized water to bring the volume to 25 µl. The program of the first PCR was 96ºC for 4 min; 30 cycles of 96ºC for 10s, 50ºC for 30s, and 72ºC for 1min; and finally, 72ºC for 2 min. The program of the second PCR was 94ºC for 4 min; 30 cycles of 94ºC for 30s, 58ºC for 30s, and 68ºC for 1min; and finally, 68ºC for 7min. PCR amplification was performed in a Flexigene thermal cycler (Techne, UK).

2.3.4 Temporal temperature gradient gel electrophoresis (TTGE) analysis

PCR products obtained fromV3 region amplification was subjected to TTGE analysis. TTGE was performed by using the Dcode Universal mutation detection system (Bio Rad. USA) that were 16cm by 16cm by 1mm. Gels were prepared with 8% (wt\vol) acrylamide stock (acrylamid : bisacrylamide 37.5:1) and a final Urea concentration of 7M. TTGE parameters and gradient temperature were optimized to separate the bacterial species (with low -G+C-content genomes). The final electrophoresis condition were 41 V for 16h with an initial temperature 63ºC and final temperature 70ºC (the temperature was increased 0.4ºC per hour). After runs gels were stained for 25 min with ethidium bromide. Gels photographed were converted into a file image (Digi image–Majour Science- Taiwan) and analyzed using

Gel ComparII version 5.00 software (Applied Maths, Kortrijk, Belgium).Data are presented as the mean ± standard deviation, and n represents the number of the tested samples.

2.4 Statistical Analysis

Data are presented as the mean ± standard deviation, and n represents the number of samples from the raw milk and the control.

3. RESULTS AND DISCUSSION

3.1 Standard Plate Count (SPC)

The rate of microbial contamination of cow's raw milk is influenced by the health status and hygiene of dairy cows, hygiene of the environment in which dairy cows are housed and milked, methods of udder preparation and milking techniques, methods used for the cleaning and disinfection of milking machines and milk tanks and hygiene of the attendant staff. Other important factors are the rate of milk chilling to the required temperature and the length of milk storage [16].

The obtained bacterial count results from twenty one samples of raw milk were represented in tables 1 to 4. The highest total viable count ($5.8x10^7$cfu/ml) in PCA was recorded in summer's sample M15. While less total viable count were observed in samples M4, M13 and M21 in winter, spring and autumn, where the total count were $3.2x10^5$, $3.0x10^5$ and $3.5x10^5$cfu/ml, respectively. Aaku et al. [17] and Arenas et al. [18] observed that the total numbers of micro-organisms in pooled raw milk were $5.5x\ 10^6$cfu/ml and 10^6 to 10^7 cfu/ml, respectively. In which these values were almost at the same limit of our experiment results.

Also the highest count of *Staphylococcus aureus* was $8.5x10^5$cfu/ml in VJA (was detected in sample M15). Whereas, the highest count of spore forming bacilli was $4.0x10^5$cfu/ml in NA in winter's sample M9. Also the highest count of *Entercoccus* sp. in SF and *Salmonella* sp. in XLDA were detected in the same sample ($4.5x10^4$ and $5.25x10^4$cfu/ml), respectively.

The highest coliform count was recorded in summer's sample M15 ($5.8x10^5$cfu/ml) in VR agar. Leitner et al. [19] and Bramley and McKinnon [1] reported a correlation between the number of coliform and psychrotrophic micro-organisms, as some species of the genera making up the coliform group of bacteria are psychrotrophic and constitute 10–30% of the whole group of micro-organisms. With *E. coli* being an important member of the coliform bacteria, its presence suggests that other enteric pathogens like S*almonella* sp. and S*higella* sp. may also be present in the test sample. Some strains of *E.coli* can cause gastroenteritis; urinary tract infection as well as diarrhea in infants. Coliform bacteria can be carried into milk duct of the cow during milking by suction of the milking machine and then flushed out during subsequent milking without causing clinical symptoms of infection. Previous studies provided evidence that *Escherichia coli* are frequently occurring organism in milk. The methods of production, transportation, handling and sale of milk are entirely unhygienic. The rate of microbial contamination of milk by mesophilic and psychrotrophic bacteria is mainly influenced by the level of herd hygiene and by the observation of hygienic principles of milk acquirement and storage. Also adequate care, treatment of the animals, and regular check up of the animals in the farms affected on the occurrence microorganisms according to [20] differences in feeding and housing strategies of cows may influence the microbial quality of milk.

3.2 Culture Independent Method

TTGE and DDGE are now methods that have been used to determine the genetic diversities of natural microbial communities such as the communities in biofilms [21], soil [22], and fermented foods [23]. PCR-TTGE enabled the dominant bacterial species present in the raw milk to be highlighted and microbiota dynamics to be observed in our batches. Major visible species represented from 1% to 100% of the total flora [24,25] Fig. (1) showed identification results using Temporal Temperature Gradient gel Electrophoresis (TTGE) as a culture-independent method. The obtained results revealed that *Enterococcus faeciun* (band B) was dominant specie that found in 11 raw milk samples, whereas *Staphylococcus aureus* (band A) and *Enterococcus faecalis* (band C) were a minor species that occurred in 6 and 3 raw milk samples respectively. TTGE provides a description of the dominant bacterial species in a complex ecosystem. Minority bacterial species cannot be detected if they account for less than 1% of the most dominant species. Our results are in agreement (Eleven samples from 21 milk samples) with those obtained with other complex media [9; 26,27]

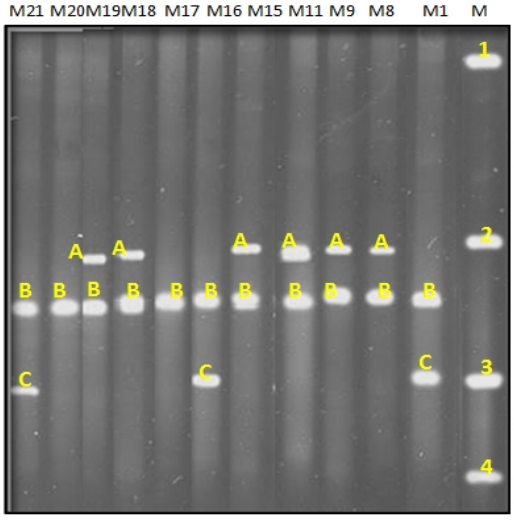

Fig. 1. TTGE pattern of V3 16S rDNA fragments from raw milk samples. M: Marker (1: *Lactococcus garvieae,* 2: *Lactococcus raffinolactis*: 3, *Enterococcus faecalis;* 4: *Lactococcus lactis* ssp. *lactis biovar diacetylactis*). After standardization of the gel by Gel Compare software, bands are identified as follow: A: *Staphylococcus aureaus*, B: *Enterococcus faecium*, C: *Enterococcus faecalis*.

Table 1. Standard plate count (SPCcfu/ml) of raw milk samples in winter

Samples Count	M1	M2	M3	M4	M5	M6	M7	M8	M9
Viable total count	3.0×106±1.01	4.1×106±0.01	3.7×106±2.0	3.2×105±1.01	5.4×106±1.1	2.4×106±1.01	4.6×106±1.0	3.1×106±1.01	4.1×106±5.01
Coliform	1.1×102±0.01	8.0×102±1.01	1.8×103±3.01	9.0×102±5.01	2.0×102±1.01	2.2×105±1. 1	5.2×104±0.01	4.1×104±1.01	5.0×104±3.01
Staphylococcus aureus	3.6×102±3.01	4.5×102±2.01	1.0×103±4.01	1.2×103±2.01	4.2×103±2.01	2.9×103±1.01	2.7×102±0.02	1.3×105±3.01	3.0×105±1.6
Salmonella spp.	1.5×103±1.01	1.8×102±0.01	2.1×102±0.01	1.2×102±3.01	5.2×102±4.01	6.6×103±0.01	5.0×104±1.05	2.3×103±5.01	5.2×104±1.04
Spore forming bacilli	1.6×104±3.01	1.3×103±1. 1	1.7×103±1.01	1.6×104±1.1	7.0×103±1.01	2.0×102±1.1	2.0×102±0.04	9.0×103±1. 1	4.0×105±0.01
Enterococcus spp.	1.0×104±2.0	7.9×102±1.01	4.2×103±3.01	NIL	8.0×102±1. 1	5.9×102±1.01	2.4×102±1.02	1.1×103±1.3	4.5×104±1.04

Data are presented as mean ± SD; M1 to M9 different samples in winter.

Table 2. Standard plate count (SPCcfu/ml) of raw milk samples in spring.

Samples Count	M10	M11	M12	M13
Viable total count	$5.9\times 10^{6}\pm2.0$	$2.4\times10^{6}\pm0.1$	$6.7\times10^{5}\pm0.05$	$3.0\times10^{5}\pm2.0$
Coliform	$2.6\times10^{4}\pm0.01$	$4.8\times10^{5}\pm2.0$	$18\times10^{3}\pm0.02$	$9\times10^{2}\pm1.01$
Staphylococcus aureus	$2\times10^{4}\pm3.01$	$4.1\times10^{5}\pm2.01$	$1.0\times10^{3}\pm2.0$	$1.2\times10^{3}\pm0.01$
Salmonella spp.	$1.5\times10^{3}\pm0.02$	$1.8\times10^{3}\pm0.01$	$2.1\times10^{2}\pm1.01$	$1.2\times10^{2}\pm2.0$
Spore forming bacilli	$7.5\times10^{2}\pm0.03$	$1.8\times10^{3}\pm2.03$	$1.7\times10^{2}\pm3.02$	$1.6\times10^{3}\pm0.04$
Enterococcus spp.	$7.6\times10^{3}\pm1.01$	$3.5\times10^{4}\pm2.1$	$4.2\times10^{3}\pm2.0$	NIL

Data are presented as mean ± SD; M10 to M13 different samples in spring.

Table 3. Standard plate count (SPC cfu/ml) of raw milk samples in summer

Samples Count	M14	M15	M16	M17
Viable total count	$7.5\times10^{6}\pm2.0$	$5.8\times10^{7}\pm2.0$	$3.9\times10^{6}\pm2.0$	$7.0\times10^{6}\pm1.0$
Coliform	$3.5\times10^{4}\pm1.0$	$6.8\times10^{5}\pm2.0$	$2.8\times10^{3}\pm3.0$	$3.1\times10^{4}\pm0.1$
Staphylococcus aureus	$3.5\times10^{4}\pm2.1$	$8.5\times10^{5}\pm3.0$	$3.4\times10^{3}\pm2.0$	$4.4\times10^{3}\pm3.0$
Salmonella spp.	$3.5\times10^{3}\pm0.1$	$8.8\times10^{3}\pm2.0$	$4.1\times10^{2}\pm1.0$	$2.2\times10^{2}\pm2.0$
Spore forming bacilli	$2.5\times10^{3}\pm3.0$	$5.8\times10^{4}\pm1.0$	$2.8\times10^{3}\pm2.0$	$8.5\times10^{3}\pm1.0$
Enterococcus spp.	$8.6\times10^{3}\pm1.0$	$2.8\times10^{4}\pm0.1$	$3.8\times10^{3}\pm3.0$	$4.2\times10^{3}\pm1.0$

Data are presented as mean ± SD; M14 to M17 different samples in summer.

Table 4. Standard plate count (SPCcfu/ml) of raw milk samples in autumn

Samples Count	M18	M19	M20	M21
Viable total count	$6.0\times 10^{6}\pm0.01$	$2.4\times10^{6}\pm1.01$	$2.5\times10^{6}\pm2.0$	$3.5\times10^{5}\pm1.\ 1$
Coliform	$3.0\times10^{4}\pm0.\ 1$	$4.0\times10^{5}\pm0.01$	$1.5\times10^{3}\pm1.\ 1$	$8.5\times10^{2}\pm2.01$
Staphylococcus aureus	$2.5\times10^{4}\pm0.01$	$3.4\times10^{5}\pm0.01$	$5.9\times10^{3}\pm3.0$	$3.5\times10^{3}\pm0.\ 1$
Salmonella spp.	$2.4\times10^{3}\pm1.0$	$2.8\times10^{3}\pm0.\ 1$	$7.5\times10^{2}\pm1.01$	$3.4\times10^{2}\pm0.01$
Spore forming bacilli	$8.5\times10^{2}\pm0.01$	$2.8\times10^{3}\pm3.01$	$3.8\times10^{2}\pm0.01$	$2.3\times10^{3}\pm1.2$
Enterococcus spp.	$3.6\times10^{3}\pm0.\ 1$	$1.2\times10^{4}\pm2.\ 1$	$4.2\times10^{3}\pm0.\ 1$	$1.5\times10^{3}\pm1.0$

Data are presented as mean ± SD; M18 to M21 different samples in autumn.

4. CONCLUSION

Although milk and dairy products are important components of a healthy diet, if consumed unpasteurized they can be risky for health due to possible contamination with pathogenic bacteria. These bacteria can originate even from clinically healthy animals from which milk is derived or from environmental contamination during collection and storage of milk. Pasteurization is the most effective method of enhancing the shelf life of milk. Despite concerns to the contrary, pasteurization does not change the nutritional value of milk., this study recommended that TTGE is an excellent tool for describing the bacterial population in raw milk ecosystems. The establishment of a molecular fingerprint could be of considerable interest to industry, especially as the method is inexpensive and the setup is simple**.** On the other hand, the lack of reference pathogenic bacteria was the obstacle to identify a large game of pathogenic isolates using this technique. So, we recommend using Real Time PCR for confirming the isolates that identified using biochemical results.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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