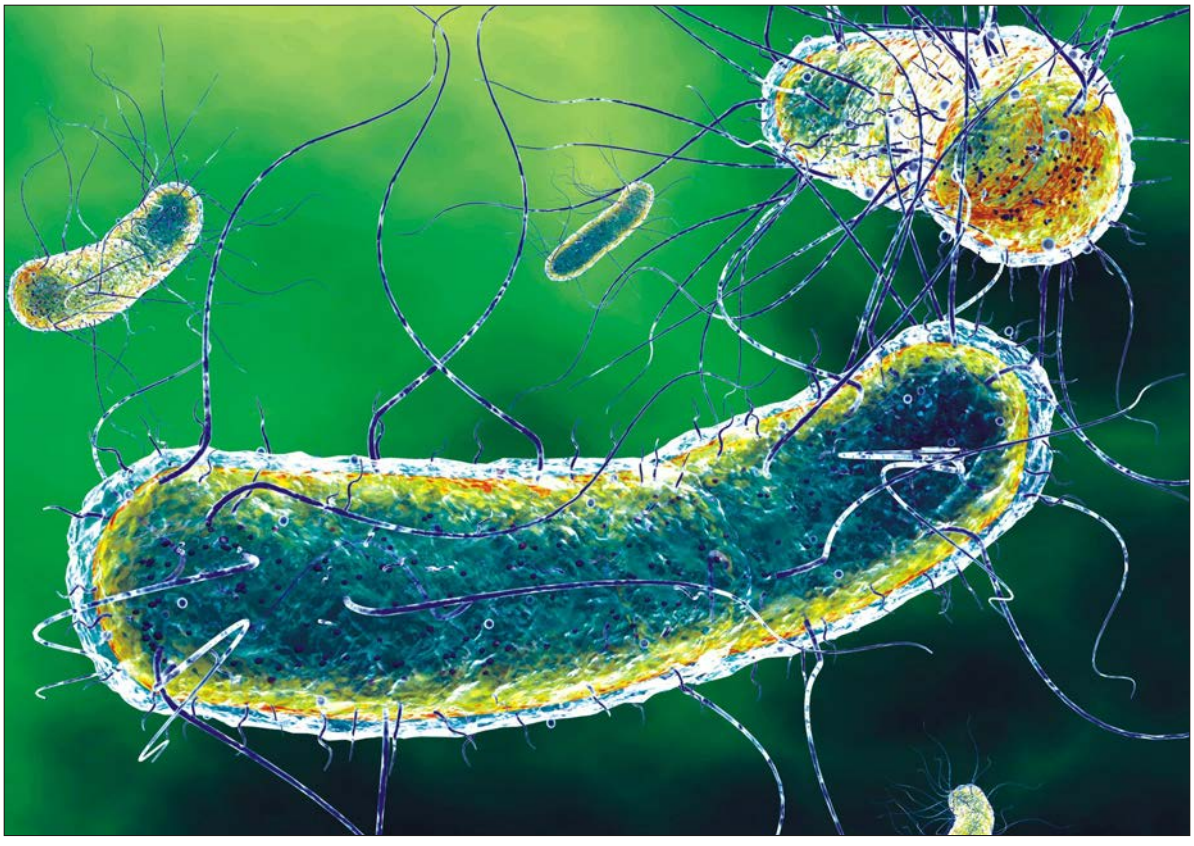

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Molecular and Bacteriological Examination of Milk from Different Milch Animals with Special Reference to Coliforms

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Abstract: In the present study, 20 samples of raw milk of cow, buffalo and goat and pasteurized milk were collected from the local market and were analyzed for microbial count and IMViC tests to determine the coliform load in the sample. Further, the presence of *E. coli* was confirmed by using PCR. Majority of the milk samples of different origin were found to be contaminated by the coliform group of bacteria. Nine samples were found to be positive for *E. coli* by PCR analysis. Pasteurized milk samples did not showed presence of *E. coli* by PCR, but they showed considerable count of bacterial growth by total plate count method. The results indicated that analyzed milk could contribute a potential risk for public health in the cases that it was consumed or used in the production of dairy products without being pasteurized or being subjected to a sufficient heat process. Moreover, PCR is less labor intensive and more rapid for bacterial identification.

Key words: Milk, coliform, PCR, *E. coli*, bacteriological analysis, milch animals

INTRODUCTION

Milk is an important food of diet of vast population on earth, due to its high nutritional value for human beings. Milk is an excellent growth medium of microorganism when suitable temperature exists. If it is produced unhygenically and handled carelessly, it gets contaminated very easily leading to its early spoilage (Oliver *et al*., 2005). Many milk-borne epidemics of human diseases have been spread by contamination of milk by spoiled hands of dairy workers, unsanitary utensils, flies and polluted water supplies. The same thing can be said for improper handling of foods in the home, restaurants, hospitals and other institutions.

The quality of milk is determined by aspects of composition and hygiene. Due to its complex biochemical composition and high water activity milk serves as an excellent culture medium for the growth and multiplication of many kinds of microorganisms.

The contamination of milk and milk products is largely due to human factor and unhygienic conditions. Usually milk gets contaminated with different kinds of microorganisms at milk collecting places. Milk is a major part of human food and plays a prominent role in the diet. Approximately 50% of the milk produced is consumed as fresh or boiled, one sixth as yoghurt or curd and remaining is utilized for manufacturing of indigenous varieties of milk products such as Ice cream, Butter, Khoa, Paneer, Rabri, Kheer, Burfi and Gulabjaman. The manufacture of these products is based on traditional method without any regard to the quality of raw material used and/or the hygienic quality of the products. Under such conditions many microorganisms can find access to the milk products.

Coliforms are considered as normal flora of intestinal tract of human and animals. They have been used as indicator organisms for bacteriological quality of milk and its products (Chatterjee *et al*., 2006).

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Coliform count is always being taken as a definite index of fecal contamination of milk and its products, that besides the possible presence of enteric pathogens which may constitute health hazards to the consumers. The most important index of microbiological quality is total bacterial count, coliforms, yeast and moulds count and detection of specific pathogens and their toxins (Szita *et al*., 2008).

Among all micro-organisms *Escherichia coli* is frequently contaminating organism in food and is reliable indicator of fecal contamination and generally present due to insanitary conditions of water, food, milk and other dairy products (Jayarao and Henning, 2001). Recovery of *E. coli* from food is an indicative of possible presence of enteropathogenic or toxigenic micro-organism which could constitute a public health hazard. Enteropathogenic *E. coli* (EEC) can cause severe diarrhea and vomiting in infants and young children. Coliforms particularly *Escherichia coli* are frequently used in the microbiological analysis of food as an indicator of poor hygienic condition.

Microbiological examination of milk is essential to find the degree of contamination and enumeration of indicator organisms. The coliform bacteria are able to grow well in a variety of substrates and to utilize a number of carbohydrates and some other organic compounds as food for energy and a number of fairly simple nitrogenous compounds as a source of nitrogen. The coliform group of bacteria is defined as the indicator (faecal coliform) of suitability of milk for drinking (Wells *et al*., 1991). The present study has been designed to assess the milk quality of different milch animals with special reference to coliforms.

MATERIALS AND METHODS

Bacterial Strain Used

Escherichia coli ATCC 11775 was obtained from American Type Culture Collection and was used in this study as reference strain (control). It was activated in luria broth at 150 rpm for 24 h at 37°C. After that, activated culture was preserved at -20°C for further use. Whenever needed, *E. coli* culture was activated in luria broth.

Sample Collection

Total twenty samples of raw milk of cow, buffalo and goat and pasteurized milk were collected from local vendors and farmers of Vallabh Vidyanagar and Anand in the month of April, 2006. Raw milk samples were collected from individual cows, buffalos and goat into sterile screw cap tubes and were directly transported to the laboratory and processed within 3 h of collection. All the chemicals used in this study were purchased from Titan Biotech, India. The cow milk samples were labeled as C1 to C5, buffalo milk samples from B1 to B5, goat milk samples from G1 to G5 and pasteurized milk samples from P1 to P5.

The study was carried out in two stages. Milk samples from different milch animals and pasteurized milk samples were analyzed during the first stage by agar plate and sugar fermentation methods. In the second stage, confirmation of bacteria was carried out by Polymerase Chain reaction.

Yeast and Mold Count of Milk Samples

All the microbiological tests were performed according to Bacteriological Analytical Manual (1998). Potato Dextrose Agar (PDA), pH 5.6±0.2 was used for Yeast and Mold count. 10^{-1} and 10^{-2} dilutions of the milk samples were prepared. Then 0.1 mL of aliquote was taken from each dilution and spread on the solidified PDA plates. These plates were incubated at 25°C for 4-5 days.

Gas Production from Lactose

This test was performed to determine presence of coliforms in the sample. The samples were inoculated in a tube of LST (Lauryl Tryptose Braoth) and incubated for 48±2 h at 35°C. Gas

production (displacement of medium from inner vial) or effervescence after gentle agitation is considered positive reaction. Positive tubes showing gas production were confirmed for coliform presence by transferring their contents to test tubes with the same culture broth and to Eosine Methylene Blue (EMB) agar plates. Streaking of culture liquid with platinum loops was used as the culture method. Gram staining of all the samples was carried out. All cultures appearing as Gram-negative, short rods were tested for the IMViC reactions and also re-inoculated back into LST to confirm gas production.

IMViC Tests

Test for Indole Production

The diluted samples were inoculated in the tube of tryptone broth and incubated at 24±2 h at 35°C. 0.2-0.3 mL of Kovac's reagent was added into the tubes. Appearance of distinct red color in upper layer is the positive test.

Voges-Proskauer (Vp)-Reactive Compounds

The diluted samples were inoculated into tube of MR-VP broth and incubated for 48±2 h at 35°C. After transferring 1 mL content into another tube, 0.6 mL α-naphthol solution and 0.2 mL 40% KOH were added. Few crystals of creatine were added. It was mixed well and allowed to stand for 2 h. Test is positive if eosin pink color develops.

Methyl Red-Reactive Compounds

After VP test, MR-VP tubes were incubated for additional 48±2 h at 35°C. After that, 5 drops of methyl red solution was added to each tube. Distinct red color is positive test. Yellow is negative reaction.

Citrate

The diluted milk samples were inoculated into the tube of Koser's citrate broth and incubated for 96 h at 35°C. Development of distinct turbidity is positive reaction.

Total Plate Count

Nutrient Agar; pH 7.4±0.2 was used for coliform count. The process was similar to that followed for Yeast and Mold count. These plates were incubated at 37°C for 48 h.

Violet Red Bile Agar Method

Violate Red Bile Agar (VRBA); pH 7.4±0.2 was used for coliform count. The process was similar to that followed for Yeast and Mold count. These plates were incubated at 37°C for 48 h.

Brilliant Green Bile Broth

Subsequently, positive growth on plates were inoculated into Brilliant green Bile Broth (BGLB) and incubated at 37°C for 48 h for complete test. Gas production in BGLB was used for the detection of coliforms after 48 h incubation.

DNA Isolation and PCR Assay

Colonies which grow on nutrient agar plate were further inoculated in to luria broth for enrichment and allowed to incubate at 160 rpm for 24 h at 37°C in orbital mechanical environment shaker. Cultures were confirmed as *E. coli* by growth on Eosin Methylene Blue (EMB) agar and testing for the formation of indole. DNA isolation from these colonies was carried out according to Sambrook *et al.* (1989). Primers used in this study were ECPAL-L 5'-GGCAATTGCGGCATGTTCTTCC-3' and ECPAL-R 5'-CCGCGTGACCTTCTACGGTGAC-3'.

The primer pair ECPAL-L and ECPAL-R is specific for all *Escherichia coli* and will amplify a control fragment of 280 bp from the exeC gene (PAL protein) (Kuhnert *et al*., 1995). DNA isolated from *Escherichia coli* ATCC 11775 was used as positive control.

Two microliter of DNA template was added to 1.25 μL 10X buffer, 2 μL 25 mM $MgCl_2$, 1 μL DNTPs mix, 1 μL each of forward and reverse primer and 0.5 μL -Taq DNA Polymerase. Total volume made upto 50 μL by adding nuclease free sterile distilled water. PCR amplification was carried out using a PCR thermal cycler (Corbett Research, Australia). The temperature profile used is as follows: Initial denaturation for 3 min at 94°C, followed by 35 cycles for 30 sec at 94°C (denaturation), 30 sec at 60°C (annealing) and 5 min at 72°C (extension). Ten microliter of the cycled PCR reaction was analyzed on a 1.5% agarose gel containing 500 ng mL^{-1} ethidium bromide at 110 V for 2 h in TAE-buffer (1 L: 5.4 g Tris, 0.4 g EDTA, 2.75 g Boric acid).

RESULTS

Out of 20 milk samples from different milch animals, 9 samples were able to ferment lactose with gas production and appeared as Gram negative rods. Table 1 shows the microbial analysis of milk samples. Log cfu mL^{-1} count on VRBA ranged from 2.153 to 2.873. For total plate count, log cfu mL^{-1} ranged from 5.665 to 4.054. Samples C3, C5, B1, B2, B4, B5, G2, G4 and G5 showed gas production on BGLB and LST, indicating the presence of coliform. Results from ImViC tests are presented in Table 2. Indole positive bacteria such as *Escherichia coli* produce tryptophanase, an enzyme that cleaves tryptophan, producing indole and other products. When Kovac's reagent (p-dimethylaminobenzaldehyde) is added to a broth with indole in it, a dark pink color developed (B.A.M.). The Methyl Red (MR) and Voges-Proskauer (VP) tests were read from a single inoculated tube of MR-VP broth. After 24-48 h of incubation the MR-VP broth was split into 2 tubes. One tube was used for the MR test, the other was used for the VP test. MR-VP media contains glucose and peptone. All enteric bacteria oxidize glucose for energy; however the end products vary depending on bacterial enzymes. Both the MR and VP tests were used to determine what end products result when the test organism degrades glucose. *E. coli* is one of the bacteria that produce acids, causing the pH to drop below 4.4. When the pH indicator methyl red is added to this acidic broth it will be cherry red, a positive MR test (BAM).

Table 1: Microbial analysis of milk samples

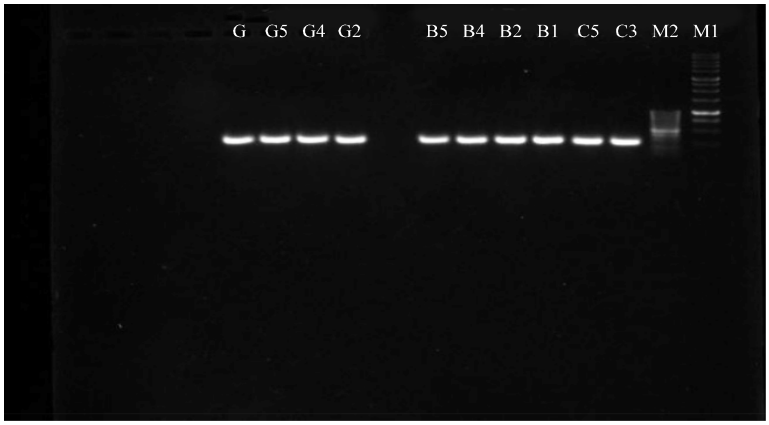
Sample No.	Yeast and mold count (log cfu mL^{-1})	VRBA (log cfu mL^{-1})	BGLB	Total place count (log cfu mL^{-1})	LST
C1	--	2.681	--	4.451	--
C2	--	2.481	--	4.574	--
C3	--	2.571	+ve	5.344	+ve
C4	--	2.670	--	4.083	--
C5	--	2.417	+ve	4.658	+ve
B1	--	2.228	+ve	4.394	+ve
B2	--	2.487	+ve	4.412	+ve
B3	--	2.521	--	4.783	--
B4	--	2.510	+ve	4.107	+ve
B5	--	2.282	+ve	4.931	+ve
G1	--	2.571	--	5.274	--
G2	--	2.873	+ve	4.201	+ve
G3	--	2.770	--	5.655	--
G4	--	2.576	+ve	4.772	+ve
G5	--	2.487	+ve	3.326	+ve
P1	--	2.521	--	4.871	--
P2	--	2.631	--	4.054	--
P3	--	2.471	--	4.476	--
P4	--	2.153	--	4.264	--
P5	--	2.768	--	4.815	--

cfu: Colony forming unit; --: Absent; +ve: Present

Table 2: IMViC test of milk samples

Sample No.	Indole production	VP	Methyl red	Citrate
C1	-ve	-ve	-ve	-ve
C2	-ve	-ve	-ve	-ve
C3	+ve	-ve	+ve	-ve
C4	-ve	-ve	-ve	-ve
C5	+ve	-ve	+ve	-ve
B1	+ve	-ve	+ve	-ve
B2	+ve	-ve	+ve	-ve
B3	-ve	-ve	-ve	-ve
B4	+ve	-ve	+ve	-ve
B5	+ve	-ve	+ve	-ve
G1	-ve	-ve	-ve	-ve
G2	+ve	-ve	+ve	-ve
G3	-ve	-ve	-ve	-ve
G4	+ve	-ve	+ve	-ve
G5	+ve	-ve	+ve	-ve
P1	-ve	-ve	-ve	-ve
P2	-ve	-ve	-ve	-ve
P3	-ve	-ve	-ve	-ve
P4	-ve	-ve	-ve	-ve
P5	-ve	-ve	-ve	-ve

+ve: Positive; -ve: Negative



M1: 100 bp marker; M2: 10 bp marker; C: Control

Fig. 1: Samples from different milch animals showing amplification by *E. coli* specific primers

The pasteurized milk samples showed presence of bacteria. Gruetmacher and Bradley (1999) cited that factors that limit the shelf life of refrigerated pasteurized milk and the microbial quality of raw milk are time and temperature of pasteurization, presence and activity of post pasteurization contaminants, types and activity of pasteurization resistant microorganisms and the storage temperature of milk after pasteurization.

PCR Analysis

PCR analysis of milk samples is shown in the Fig. 1. Samples C3, C5, B1, B2, B4, B5, G2, G4 and G5 showed amplification by the primers specific to *E. coli*. Approximately 150 bp fragment was amplified by the primers. Amplification was observed in two samples of cow milk, four samples of buffalo milk and three samples of goat milk. Pasteurized milk samples did not show any amplification. This might due to the pasteurization time and temperature at which majority of the heat sensitive microorganisms get destroyed.

DISCUSSION

Pathogenic bacteria in milk have been a major factor for public health concern since the early days of the dairy industry. Many diseases are transmissible via milk products. Traditionally raw or unpasteurised milk has been a major vehicle for transmission of pathogens (Vasavada, 1988). The health of dairy herd, milking conditions is basic determinant of milk quality. Another source of contamination by microorganisms is unclean teats. The use of unclean milking and transport equipment contributed also to the poor hygienic quality (Bonfoh *et al*., 2003). As a result of present study, the samples of raw milk examined contained coliform group of microorganisms. This may indicate that analyzed milk can contribute a potential risk for public health in the cases that it is consumed or used in the production of dairy products such as cheese, butter, cream and ice cream without being pasteurized or being subjected to a sufficient heat process.

From the results of the present study, it was found that majority of the samples were contaminated by coliform bacteria. Coliform bacteria were found in milk samples of different origin of milk. In operational conditions, mainly a failure to observe the hygienic rules of milking process contributes to the impairment of microbial quality of milk (Jayarao *et al*., 2004). Tondo *et al*. (2000) reported that 35.2% of food handlers were asymptomatic carriers of *Staphylococcus aureus* and that 90.4% of raw milk samples among more than 3200 investigated dairy products.

Oksuz *et al*. (2004) reported *E. coli* 0157: H7 at the rate of 1% in 100 samples of raw milk. Soomro *et al*. (2002) isolated *E. coli* in 57% of the 100 raw milk samples. Coliform organisms and *S. aureus* are good indicators of the standard of hygiene and handling. According to Harrigan and McCance (1976), coliform bacteria count should be less than 200 cfu g^{-1} in food. The existence of the coliforms has been considered as leading to the fact that the product was subject to process under inefficient hygienic conditions (Harrigan and McCance, 1976; Altug and Bayrak, 2003).

The high level of coliform of the fresh farm milk can indicate the evidence of unhygienic conditions of the product. Collins *et al*. (1995) reported that *E. coli* and coliform bacilli which they are belong the family of Enterobacteriaceae may indicate evidence of contamination or pollution especially of fecal nature. Enterobacteriaceae include other organisms, like important pathogens such as salmonella and various non-lactose fermenters that may be present in human and animal faeces. The bacterial count of milk is used to measure its sanitary quality and most grading of milk is on the basis of some method for estimating numbers (Collins *et al*., 1995).

Post pasteurization contamination has received most of the attention and is considered to be the factor, which limits shelf life in the majority of cases (Waes, 1982). Pasteurized milk, which was collected from the shops, showed different values for standard bacterial counts. The higher count of coliform bacteria in the milk might be due to improper handling, poor cleaning and storage of equipments as stated by Hayes *et al*. (2001).

The total viable count of fresh milk samples of this research showed a mean value of 4.572 log cfu mL^{-1}. Milk can be contaminated with different kind of microorganisms due to direct or indirect contact with any source of external contamination during the steps of milking, collection, packing and transport. Direct physical contact of milk with unclean surfaces such as those of milking utensils, udders and teats and the hands of milkers besides environmental factors such as the design and cleanliness of buildings and installations, the adequacy of the water supply, the manner in which the manure and other wastes are disposed of and the amount of dust in the immediate surroundings are important in so far as they may contribute to the microbial contamination of surfaces with which milk comes in to contact.

During milking operation, however, milk may be exposed to contamination from the animal, especially the exterior of the udder and adjacent areas. Bacteria found in manure, soil and water may enter from this source. Such contamination can be reduced by clipping the cow and washing the udder

with water or a germicidal solution before milking. Contamination of cow with manure, soil and water may also be reduced by paving and draining barnyards, keeping cows from stagnant pools and cleaning manure from the barns or milking parlors. Pasteurization kills pathogens that may enter the milk and improves the keeping quality of milk.

Recently, PCR methods are mostly used for the detection of microorganisms in different types of food materials. These methods often allow superior specificity to traditional biochemical identification methods. In the present study, colonies growing on the Nutrient agar plate were given pre-enrichment. This pre-enrichment step was performed to achieve appropriate sensitivity. These pre-enrichment protocols also greatly reduce the risk of false positive results due to detection of killed organisms, mainly due to the sample dilution step inherent to these protocols. Another critical component of the PCR assay is the inclusion of an internal positive control that indicates PCR failures, e.g., through carry over of PCR inhibitors. Differentiation of bacterial foodborne pathogens beyond the species level also provides exciting opportunities to better understand the biology of bacterial strains and subtypes, including differences in their ability to cause human foodborne disease.

Samples analyzed in the present study can contribute a potential risk for public health in the cases that it is consumed or used in the production of dairy products such as cheese, butter, cream and ice cream without being pasteurized or being subjected to a sufficient heat process. Moreover, PCR is less labor intensive and more rapid than bacteria culturing and conventional methods of bacterial identification.

Information given by the obtained results allow to conclude that strict hygienic measures should be applied during production, processing and distribution of milk and it's products to avoid contamination. Periodical inspection must be done by specialists on the dairy farms to minimize milk contamination with different types of microorganism. Efficient cleaning and sanitization of farm dairy utensils must be done to improve the quality of raw milk and consequently the related dairy products. The milk and milk products should be kept under refrigeration at all times and the practice of display at room temperature should be discouraged.

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