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Research Article

Detection and Analysis of Pathogenic Bacteria from Raw and Pasteurized Milk

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Abstract

Milk is a proper and complete healthy diet for all individuals. Milk contains all the basic nutrients such as carbon, calcium, and rich water content which facilities bacterial contamination if hygienic conditions are not properly maintained. Different types of bacteria such as gram-positive and gram-negative can contaminate milk in many ways such as use of unclean utensils, storage tanks, and other factors which includes diseased animals, or/and animals suffering from mastitis. Most commonly isolated organisms from raw milk samples are *E. coli, Shigella, Staphylococcus aureus, Klebsiella*, and spores forming Bacillus species which can cause serious enteric illness if contaminated consumed. Pasteurization process extensively reduces the risk of contamination and makes the milk safer for drinking. In this study, we have collected 24 samples in total of raw milk (n=12) and pasteurized milk (n=12). Further, we processed the milk samples on different agars. For raw milk, we used Nutrient agar, Mannitol salt agar, and Eosin Methylene blue agar whereas for pasteurized milk we have used dye reduction test. Raw milk indicated high contamination as compared to the milk which is pasteurized. Even some locally available pasteurized milk has detected contaminating agents. As the results indicated that raw milk is highly contaminated so for the safety of individuals and the population, it is suggested that we should use pasteurized milk in our daily life. Furthermore, we must apply some safety precautions including maintenance of tanks, storage temperature, and sterilization of utensils in which milk is kept.

Introduction

Milk is extremely healthy food for all individuals. Milk of cattle, buffalo, goat, and sheep contains the same but different concentrations of a chemical constituent AU-Hahn et al. [1]. A vast variety of products are made from milk such as cheese, yogurt, butter, cream, and other dairy food. The nutritional complex of milk is extremely multifaceted. It contains all the main molecules which are required for the body and bone development. One cup of whole cow's milk contains calories, proteins, 88% water, carbohydrates, sugar, and fiber Langiano E et al. [2]. All these compounds make the milk, an excellent source of high-quality food for all age groups Elgohary et al. [3]. A carbohydrate makes up around 5% of milk usually in the form of lactose sugar which some individuals cannot digest Mohammad-Mehdi Soltan et al. [4]. In milk, some vitamins are also present such as vitamin B12, vitamin D, and calcium, riboflavin, phosphorus. Clean milk is drawn from a healthy cow that has a low microbial load and good for drinking but when milk is stored at a low temperature, it can be spoiled quickly and contaminated with bacteria. We can say unpasteurized milk acts as the best medium for microbial development Uddin et al. [5].

Raw milk comes from cows, goats in farms which is free from microorganisms, but the treatment process of milk can make it harmful or unhealthy Boycheva S et al. [6]. Raw milk is contaminated with various sources such as a closed milking system, improper bulk tanks, unhygienic storage equipment, and unclean processing area and other factors Mungai EA et al [7]. Various types of bacterial species can contaminate milk in different ways which includes fecal contaminated organisms, gram-positive, and gram-negative etc. Oliver et al. [8].

Pasteurization is a process in which raw milk is treated at high temperatures to reduce or eliminate the bacteria from milk and make the milk safer for consumers Desmasures N et al. [9]. Pasteurized milk is considered to be safe for a person because the

heat treatment process kills the bacteria and other organisms such as *salmonella, Campylobacter, E. coli*, etc Yoti et al. [10]. Pasteurized milk does not reduce the nutritive value of milk. Basically, in the pasteurization process, heating of milk and quick cooling leads to the elimination of certain bacteria. For best results, pasteurized milk heated at a high temperature of 145 degrees Fahrenheit for 30 minutes. This method is also called batch-pasteurization, which is not commonly available Banwart GJ et al. [11].

There is another method is the Ultra-Heat Treatment method which involves very high temperatures and less time such as milk is treated with high heat 280 degrees Fahrenheit for 1 to 2 seconds Campos MRH et al. [12]. This temperature extended shelf life for up to 9 months. Another method is flash pasteurization, in which a liquid milk sample is heated for 15 minutes at 161.6 degrees Fahrenheit Chatterjee SN et al. [13]. This method is also known as high-temperature short time.

In the early 20th century, there were no proper instructions for temperature and heating time to get rid of the bacteria from milk. But in 1943, HTST (high-temperature short time) came in use Carvalho PLN et al. [14]. In this method, 72 °C is used for 15 seconds while in batch pasteurization, 63 °C is used for 30 minutes to maintain the thermal death time (the temperature which is required for complete killing of bacteria) Desmasures N et al. [15].

Another important and commercialized method of pasteurization is low temperature-short time (LTST). In this method, the temperature is used for the killing of bacteria at 145 F for 30 minutes Devi et al. [16]. It is also known as vat pasteurization. This method takes a thousandth second to treat milk, so it is called millisecond technology (MST).

Milk contamination occurs from various factors such as diseased animals which are suffered from mastitis (an animal disease), from improper cleaning of milking equipment and milk tanks in which milk is collected, stored, and transported Deshmukh AM et al. [17]. The most common cause of raw milk contamination is soiled teats, udders, and tails of animals. Milk is an enrichment medium for the development of microbes Eberhart RJ et al. [18]. During the transportation of milk at normal temperature, the microbes multiply and decline the quality of milk. This study was conducted to detect and analyze the pathogenic microbes among different collected samples of raw and pasteurized milk Ekici K et al. [19].

Material and Methods

Sample collection

We have collected 24 milk samples to conduct this study. Out of which, twelve (12) raw milk samples were collected from local dairy shops, and twelve (12) pasteurized milk samples were collected of different brands from the supermarket.

After the collection, the sterility of the samples was checked.

After drawing a 1ml sample each from different bottles by placing them at refrigeration temperature. The quality of these samples was checked according to the following steps:

1. All samples were collected aseptically.
2. All the 12 samples of raw and pasteurized milk had streaked on N.A, EMB, MSA plates.
3. Plates were incubated at 37c for 24 hours.
4. The next day plates were observed, and biochemical testing had performed for further identification.

Biochemical testing

Biochemical testing was performed to differentiate bacterial species. Bacterial species were differing from each other due to some changes in carbohydrates, protein, fats metabolism, production of certain enzymes, etc., Vlaemynck G et al. [20]. There are various types of biochemical test which were performed for various bacterial species such as gram -ve isolates, IMVIC was performed.

Imvic (Indole Methyl Red Voges Prokeur Citrate)

Indole test: The indole test is mainly done for the detection of the tryptophan enzyme in which tryptophan broth was incubated with tested organisms. It was incubated at 37 °C for 24 hours. After incubation, Kovac's reagent was added to check the presence of the enzyme. If the tryptophan enzyme is present red ring is observed at the top of the layer of broth (cherry red color represents indole present).

Methyl Red: This is a biochemical test that is used to detect the formation of different acids by microorganisms such as acetic acid and formic acid by the fermentation of sugars Beerens H et al. [21]. Testing isolates were inoculated in Clark's broth and incubated at 37 °C for 24 hours. After incubation red color was observed by the addition of methyl red which indicated production or fermentation of sugars. If acid produces the color of broth change into red after adding indicator whereas the organism does not produce acid the color of broth remains yellow Pathogen Safety Data Sheets and Risk Assessment 2014 [22].

Voges Proskauer Test (VP): This test is mainly used for the determination of organisms that produces acetyl methyl carbinol as a result of glucose fermentation. Tested organisms were inoculated in Clark's broth and incubated at 37 °C for 24 hours. After incubation 1ml of broth culture was transferred in a tube, in which 0.6ml of 5% alpha naphthol followed by 0.2ml of 40% KOH were added. A positive test indicates the development of red color after 10 to 15 minutes. A negative test indicates no coloration Banwart, GJ et al. [11].

Citrate Test: The citrate test was done to detect the ability to consume citrate by an organism as the only source of carbon and energy. Tested organisms were picked from isolated pure culture and streaked on citrate agar slant and incubated at 37 °C for 24 hours. After incubation, the color of the slant was observed. When organisms metabolize the citrate the color of the slant turns into bright blue. If organisms do not utilize the citrate the color of the slant remains green.

TSI (Triple Sugar Iron Test): TSI stands for a triple sugar iron test. This analysis was performed to check the capability of a microorganism to produce hydrogen sulfide by the fermentation of available sugars. This test is made for the enteric bacteria which includes *Shigella* and *salmonella* species. This test indicates that tested organisms ferment sugars such as glucose, sucrose, and lactose that showed the production of by-products. If slant and butt both turned yellow indicate that an acidic condition was developed, and all the sugars were fermented. If alkaline slant and acidic butt show only glucose fermentation while both butt and slant show alkaline that indicates that no sugar fermentation has occurred. The blackening of the medium was indicated by the production of hydrogen gas. Carbon dioxide gas production is indicated by the cracks and bubbles formation.

Urease Test: Urease test is performed to detect the breakdown of urea by the action of an enzyme urease done by microorganisms. Streaked the isolated colony on urea slant and incubated at 37 °C for 24hours than observed the color change of slant. If urease enzyme present urea breakdown into ammonia and slant turned pink in color. If organisms were not produced urease enzymes the color of slant remains the same light orange.

Dye reduction test (methylene blue): This test was used to estimate the microbial load of pasteurized and packed milk (Table 1).

Table 1: Grading of milk according to the time in which dye decolorize.

Time	Quality
5 hours and above	Good
3 to 4 hours	Very good
1 to 2 hours	Fair
Less than ½ hours	Poor

Results

Bacterial Identification in raw milk samples

In our study different types of bacteria were identified from the raw milk samples. According to these results, the most common isolates in raw milk samples were *staphylococcus aureus, Salmonella*, and *Bacillus*. Other enteric organisms were also found like *E. coli, Klebsiella spp*, and *Proteus spp*. We performed bacterial identification on N.A, EMB, and MSA for all 12 raw milk samples. The following results were obtained that are mentioned in Table 2. These organisms are further identified on the basis of colonial and biochemical characteristics as mentioned in Table 3.

Table 2: Bacterial identification on N.A, EMB and MSA.

S.NO.	N. A	EMB	MSA
Sample 1	*B. subtilis*	*Klebsiella spp*	*Staphylococcus. aureus*
Sample 2	*B. cereus*	*E. coli*	*Staph. Aureus*
Sample 3	*B. subtilis*	*Salmonella spp*	*Staph. Aureus*
Sample 4	*B. subtilis*	*E. coli*	*Staph. Aureus*
Sample 5	*B. cereus*	*Shigella spp*	*No growth*
Sample 6	*B. subtilis*	*E. coli*	*Staph. Aureus*
Sample 7	*B. cereus*	*Salmonella typhi*	*Staph. Aureus*
Sample 8	*B. subtilis*	*Proteus vulgaris*	*No growth*
Sample 9	*B. cereus*	*E. coli*	*Staph. Aureus*
Sample 10	*B. cereus*	*Enterobacter aerogenosa*	*No growth*
Sample 11	*B. subtilis*	*E. coli*	*Staph. Aureus*
Sample 12	*B. cereus*	*E. coli*	*Staph. Aureus*

Table 3: Colonial and biochemical characteristics.

	Bacillus spp	*staph*	*E. coli*	*Shigella spp*	*Salmonella spp*	*Proteus Vulgaris*	*Enterobacter aerogenes*	*Klebsiella spp*
Colonial characters	Circular, white, Colonies	Golden colonies	Green Metallic sheen	Black, small colonies	Round, brown colonies	Round, small grey colonies	Round small black/purple color colonies	Circular mucoid pink color colonies
Microscopy	Rod-shaped chain purple in color	Bunches purple color	Rod in red color	Short rods red in color	Short rods red in color	Short rods red in color	Short rod red in color	Short rod red in color

IMVIC	+/+/-/-		+/+/-/-	-/+/-/-	-/+/-/+	+/+/-/-	-/-/+/+	-/-/+/+
TSI			A/A/-/-	A/A/-/-	A/A/+/+	A/A/+/+	A/A/-/-	A/A/-/-
Catalase	+							
Urease		-	-	-	+	+	-	-

Methylene blue reduction test

No colonial growth found on the plates of EMB, N.A, MSA. Samples were proceeded to perform M.B.R. The results obtained are mentioned in Table 4 & Figure 1A-1E.

Table 4: Qualitative analysis of Milk Sample by Methylene blue reduction test.

Samples	Reduction time	Grades
Olpers	6 hours	Excellent
Nestle milk pack	6 hours	Excellent
Nurpur	1 hour	Very poor
Nestle nesvita	5 hours	Good
Pakola milk	5 hours	Good
Day fresh	2 hours	Fair
Haleeb	6 hours	Excellent
Good milk	5 hours	Good
Omang	6 hours	Excellent
Gourmet milk	4 hours	Fair
Daily dairy	4 hours	Fair
Tarang	5 hours	Good

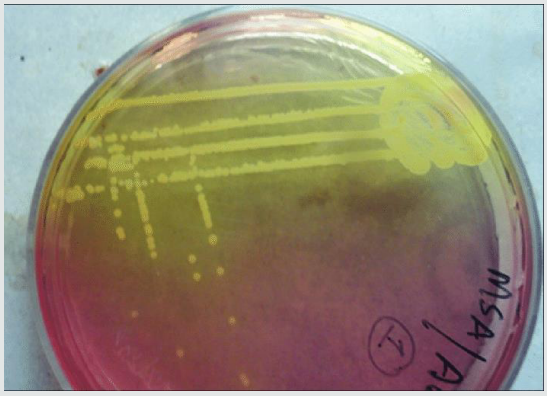

Figure 1: Colonial identification of raw milk samples.

Figure 1A: *Staph. aureus* on MSA.

Figure 1B: *E. coli* on EMB.

Figure 1C: Microscopy of *Bacillus Cereus.*

Figure 1D: TSI Result of *E. coli.*

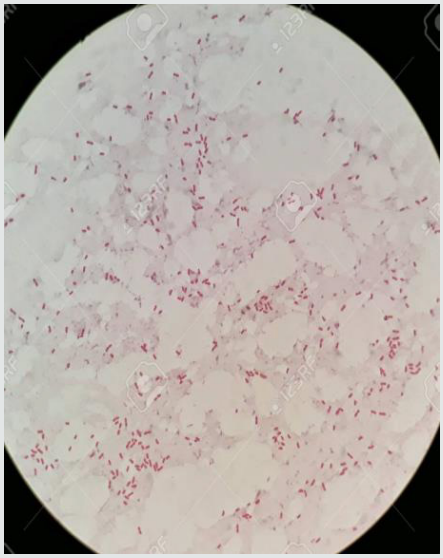

Figure 1E: IMVIC Result of *E. coli*.

Discussion

Milk is the best food for all age groups with all requirements which is necessary for individual growth and development. Different types of milk are available for the consumer to consume according to their taste and desire Karmali MA et al. [23]. Milk is composed of all basic nutrients such as calcium, phosphorus, vitamins, and water content Potter M et al. [24]. The composition of milk is considered as complete nutrition. The high amount of nutrients in milk supports the growth of microorganisms and causes spoilage of milk. High water content in milk is the basic factor for bacteria to grow Hill C et al. [25].

Raw milk is unheated (untreated milk) are readily available in the markets. Which is highly contaminated with pathogenic bacteria. Bacteria are present in the environment that's why it contaminates the milk and its products easily Czaplicki A et al. [26]. Milk becomes contaminated with different ways such as fecally contaminated water when added in the milk, unsterile utensils, improper bulk tanks, soil, and major and most dangerous factor is that unhealthy condition of milking man because hygiene of personal is very important if caretaker of cows and the milking man was not properly clean in ordered to wash their hands and if suffer from any diseases so organisms should be transferred and contaminate the milk another factor is a diseased animal Hijiumi et al, 2011. Animal health is also very important because cow suffer from mastitis and organisms enter into milk and causes spoilage Ekici k et al. [17].

According to different studies, untreated milk is not healthy for human use until it is not properly boiled. But sometimes after boiling, microorganism remains present in the milk and causes enteric diseases such as diarrhea, vomiting, etc Oliver sp et al. [21]. All these effects and health issues of raw milk increases the value of pasteurized milk. Pasteurized milk is of better quality and long shelf life than raw milk and no health issues are measure from pasteurized milk Hill C et al. [23]. Pakistan is the biggest milk-producing country in the world. Milk contains major nutrients that support human health and makes the bones strong and prevent osteoporosis, but these nutrients also support the growth of bacteria which causes contamination. According to studies typhoid fever is very common in young children.

In this study we collected 24, out of which 12 were raw milk samples from local dairy shops of Karachi city and 12 were branded and local pasteurized milk samples such as Olpers, Haleeb, Nestle Milk Pack, Nurpur, Nestle Nesvita, Pakola, Day fresh, Good milk, Omang, Gourmet milk, Daily Dairy, and Trang. This study showed that raw milk is highly contaminated with soil, unhealthy water, and a high number of pathogenic bacteria. Whereas no growth was observed in pasteurized milk.

The outcome shows that the numbers of aerobic spores are readily available in milk samples because the reported concentration of aerobic spores in grass and maize vary from 10 to >105 per g. When spores present in soil and animal feed, they form high numbers of spores these spores excreted in feces. *Bacillus* species are rich in the environment and therefore naturally present in the soil. The proportion of milk samples that showed activist for *S. aureus*, the major pathogen-related with diseased cow condition worldwide. All types of bacteria which are present in milk samples are diseases causing but some of them are not related to foodborne diseases such as Klebsiella and Proteus these organisms cause pneumonia and urinary tract infections, respectively.

In this study the presence of microorganisms in the pasteurized milk was less than raw milk, the Methylene blue reductase test was performed for the detection of the quality of milk sample, pasteurized milk samples including Olpers, nestle milk pack, Haleeb and Omang showed the excellent quality of milk with 6hrs, Pakola, good milk, Trang and nestle Nesvita indicated good value of milk with 5 hours reduction time while Nurpur, the day fresh, daily dairy and gourmet milk showed poor quality and their reduction time was 1hr to 2hr. The implementation of good sanitation practices and applications of HACCP (hazard analysis of critical control points) will improve the quality of milk and also done by increasing the temperature and timing of the heating process will help to reduce the growth of microorganisms Armstrong C et al. [27].

A study of *S. aureus* in raw and pasteurized milk was conducted in the Reconcavo area of the state Bahria, Brazil. In this study, a total of 70 samples were studied out of which 50 samples of raw milk and 20 samples of pasteurized milk were taken. Out of 50 samples of raw milk, 34 showed the contamination of *S. aureus* while out of 20

samples of pasteurized milk 6 samples contaminated with *S. aureus* with percentages of 68% and 30% respectively. This contamination showed that both types of milk were health hazardous. In Dhaka city Bangladesh, a study for the characterization of pathogenic bacteria in raw and pasteurized milk had conducted. This study showed raw milk is highly contaminated with fecal bacteria known as *Shigella, Klebsiella E. coli*, whereas pasteurized milk was slightly contaminated with different types of bacteria such as Bacillus app Uddin et al. [5].

Pasteurization quality will be increased by increasing the heating time because milk becomes contaminated by different types of microorganisms that might be reduced by plenty of milking and transport measurement. The large number of Escherichia coli in milk samples showed that fecal contamination in milk and diseased condition of cows. Proper time and temperature in the pasteurization process must be maintained to eliminate pathogens and other contamination also some immediate actions for raw milk safety should be taken because individuals may suffer from illnesses. Due to the use of raw milk because of their unawareness.

Conclusion

Raw milk and locally available milk have constantly open risk to the general public because of the possible presence of pathogenic bacteria. Internationally, outbreaks of foodborne sickness have been attributed to unprocessed milk. This can be reduced by using boiled water for cleaning all utensils and equipment. It is also preferred that detergents or disinfectants should be dissolved in boiled water for cleaning of all utensils and equipment which immediately helps to reduce milk contaminates. For pasteurized milk or locally available brands of milk, one should make sure that their processing areas are cleaned and proper which provides, ultra-high temperature to decrease bacterial contamination in milk.

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