Research Article

Biochemical and Bacteriological Analysis of Cows' Milk Samples Collected from District Peshawar

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ABSTRACT

Milk is an essential food for human beings and it also acts as a good medium for microorganism's growth. A total of 60 cows' raw milk samples were processed for bacteriological and biochemical analyses, collected from different locations of District Peshawar, Pakistan. Bacteriological analysis was performed for the detection of Coliforms including *E.coli,* and *E.aerogenes*. Tests were also performed for detection of *Salmonella.* Analysis was done on specific media, while for confirmation of bacteria, various biochemical tests were performed. Coliforms were detected in 35% milk samples in which *E.coli* were detected in 31.67% milk samples while *E.aerogenes* were detected in 26.67% milk samples. *Salmonella* was detected in lowest percentage with 6.67%. Biochemical tests were also performed for detection of various adulterants in theses milk samples using MAT (Milk adulteration Test) kit prepared by WTO Labs, UVAS, Lahore, Pakistan. Tests were performed for detection of urea, detergents, salt, starch, sugar, formalin, boric acid, carbonates, hydrogen peroxide and ammonium sulphate. Formalin was detected in highest percentage with 28.33% followed by starch which was recorded in 26.67% milk samples. The other adulterants like carbonates, sugar, salt and ammonium sulphate and boric acid were detected in 21.67%, 18.33%, 16.67% and 11.67% milk samples respectively. None of the milk sample was found positive for urea, hydrogen peroxide and detergents adulteration. It was concluded from the present study that raw cows' milk is mostly contaminated with bacteria and adulterated with different chemicals which may lead to various health hazards.

Keywords: Cow milk, Bacteriological analysis, MAT Kit, Adulterants.

INTRODUCTION

Milk and its products with high biological potential, enriched nutritional values and without health risks and hazards are generally demanded for nutritional purposes[1,2]. The compositions of raw milk differ by species, but significant amounts of saturated fats, calcium and protein as well as vitamin C is there in milk. Cow's milk is slightly acidic with pH ranging from 6.4 to 6.8[3].

Milk samples including infant formulas, milk powder, raw milk (Unprocessed), milk from markets, human milk and animal milk from various countries such as Italy, Canada, Lithuania, Poland, USA, UK and Nigeria have been studied thoroughly for the assessment of milk quality and safety[4,5].

Being an essential food for human beings, milk also acts as a good medium for the growth of many microorganisms. Microorganisms that can easily grow in milk include *Lactobacillus, Streptococcus,* Coliforms, *Staphylococcus* and *Micrococcus spp*. Microbial contamination of raw milk can occur from various sources like air, milking equipment, feed, soil, grass and feces[6]. In appropriate conditions milk can act as a carrier of disease from milking animals to human via microorganisms[7].

Contaminated raw milk may act as a source of many harmful bacteria leading to various diseases, such as undulant fever, salmonellosis, dysentery and tuberculosis. Raw milk with a bacteria count below a specified limit is known as "certified" milk and is considered healthy. However it still may contain significant number of disease producing organism. This pathogenic bacterium present in milk often causes major public health problems, especially in those individuals who use raw milk for drinking purpose. For the removal of disease producing organisms and to increase the shelf life, raw milk is given different heat treatments[8].

Raw milk usually contains microorganisms which may lead to food borne diseases[9]. Pathogens that have been investigated to be involved in food borne outbreaks associated with the consumption of contaminated milk include *Salmonella spp., Listeria monocytogenes*, *E. coli and Staphylococcus aureus.* The presence of such types of pathogenic bacteria in milk has caused many public health problems, especially for those individuals who use such contaminated milk[10]. Raw milk may also contain aerobic *mesophilic* flora like *Micrococcus, Streptococci, Microbacterium, Arthrobacter and Bacillus* [11].

The adulteration of food products specially milk is a major problem. This is how the fraudulent producers always try to cheat consumers and authorities. Adulteration affects the quality of milk and milk products. Milk adulteration is banned due to its ill effects on health. Carbonate in milk causes various gastrointestinal problems including gastric ulcer, colon ulcer, diarrhea, and electrolytes disturbance[12]. The hydrogen peroxide has adverse effects on antioxidants balance in the body, thus disturbing the natural immunity hence increasing aging[13]. Chloride in milk causes disturbance in the acid base balance in the body and also blood pH while

Ammonia in milk may causes regression, loss of acquired speech and sensory disturbances[14].

At present Pakistan is estimated to be producing large quantity of milk up to 4 million tons per year. This production is enough to fulfill the needs of massive population of 165 million who's per year consumption of milk is estimated to be about 35 million tons. Due to lack of sufficient and proper planning, transportation, cooling system, collection and distribution facilities, and large quantity of the milk is consumed in the remote areas of Pakistan. As a result of all these factors, Pakistan is importing milk powder from abroad worth millions of dollars each year which is a huge burden on its precious foreign exchange earnings[15]. The aims and objectives of the current study were to analyze the bacteriological and biochemical analysis of milk and also evaluation of different adulterants present in milk.

MATERIALS AND METHODS

In the present study, adulterants' detection study was carried out in the Microbiology Laboratory, Veterinary Research Institute (VRI) Peshawar and bacteriological analysis was carried out in Department of Microbiology, Kohat University of Science & Technology, Kohat, Pakistan.

Collection of raw cow milk Samples

Raw Cows' milk samples were collected for this study from various parts of District Peshawar, Khyber Pakhtunkhwa. The locations included Ring road, Haji camp, Gul bahar, Hashtnagri, Firdos, Charsada road, Sadar, Tahkal, Board bazaar, Hayat abad, Khyber bazar and Karhanoo market. Samples were collected from milk shops as well as various dairy farms in the study areas. 20 ml milk was taken with the help of disposable syringes and transferred into sterile screw cap test tubes. The sampled milk was transported in ice packs to laboratory for further analysis.

Preparation of serial dilution

Each sample of milk was diluted before plating. The dilutions were made in sterilized distilled water. One ml of milk from each sample was poured into 9 m1 of sterilized distilled water in a test tube to get a dilution of (1:10). From this, further dilutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were prepared. All Petri plates were labeled with dilution factor and sample number. Diluted samples were mixed thoroughly.

Isolation of different pathogens from milk samples

Coliform Test

Milk samples were mixed thoroughly and test portion was removed immediately after mixing. 1ml of milk was transferred to each of the Petri dishes. Pour approximately 12 ml of molten media (Violet red bile agar) into each inoculated Petri dishes. After pouring the media, Petri dishes were shaken clockwise and anti-clockwise to obtain sufficiently spaced colonies, plates were allowed to solidify and these were incubated for 24 hours at 30˚C in inverted position. Appearance of dark red colonies indicated the presence of coliform[16].

Detection of *Enterobacter aerogenes* and *Escherichia coli*

1 ml of raw milk diluted sample from 10^{-3} dilution was pipette into empty sterile Petri plates and 12-15ml Eoisin Methylene Blue (EMB) was poured into Petri plates. Sample dilutions and EMB were mixed thoroughly by clock wise and anti clock wise rotation of plates on flat level surface and were incubated at 35°C for 24hours. Suspected colonies of *E.coli* and *Enterobacter aerogenes* were purified on EMB by streaking.

Isolation of Salmonella

One ml of raw milk diluted sample from 10^{-3} dilution was pipette into sterile Petri plates. And 12-15 ml of Salmonella Shigella agar (SS agar) was poured into Petri plates. Sample dilutions and SS agar were mixed thoroughly by clock wise and anti clock wise rotation of plates on flat surface. Inverted solidified Petri dishes were incubated promptly for 24 hrs at 35°C. Salmonella produced colorless to pale pink, opaque, transparent or translucent colonies. Some strains produced black centered colonies. Suspected colonies of salmonella were purified on SS agar by streaking and pure cultures of *salmonella* were obtained[17].

Biochemical Tests for adulterants' Analysis

Biochemical tests were also performed for analysis of various adulterants. These tests included urea test, detergent test, salt test, starch test, sugar test, formalin test, boric acid, carbonate test, hydrogen peroxide test and ammonium sulphate test. These tests were performed on the collected milk samples with the help of MAT kit (milk adulteration test kit) prepared by WTO Labs, Lahore, Pakistan.

RESULTS

Bacteriological analysis

Among all the sixty samples, 22 samples (36.66%) were positive for bacteriological growth. Highest numbers of positive samples were noted in Charsada road's milk samples, where 4 out of 5 samples were positive for bacteriological growth followed by Hashtnagri and Karkhano market, where three samples were noted positive. While in Haji camp, Board bazaar and Khyber bazaar 2 samples were noted positive and only one sample was noted positive each from Ring road, Gulbahar, Firdos, Sadar, Tahkal and Hayatabad (Table 1: Figure 1).

Coliforms were noted in highest percentage with 35% with 31.67%. *E.coli* and 26.67% *Enterobacter aerogenes*. *Salmonella* was detected in 6.67% samples.

Coliform test

Coliforms were noted in highest percentages with 35%. Coliform were observed to be present in 21 samples. The

maximum (4) samples were observed as positive in the milk samples collected from Charsadda road, followed by Hashtnagri and Karkhano market where 3 samples showed positive result for coliform. Two samples each from board bazaar and Khyber bazaar were noted positive for coliform test, while in Ring road,Haji camp, Gulbahar, Firdos, Sadar, Tahkal and Hayatabad only one sample from each locality was observed as positive.

Enterobacter aerogenes

Enterobacter aerogenes were detected in total 16 (26.67%) samples. In samples collected from Ring road, Haji camp, Firdos, Sadar, Tahkal, Hayatabad, only one sample was recorded positive from each locality while in Hashtnagri, Charsada road, Board bazaar, Khyber bazaar and karkhano market two samples were observed positive from each locality.

Escherichia coli

Escherichia coli were detected in 19 (31.66%) samples. The maximum (4) samples were observed as positive for *E.coli* in the milk samples collected from Charsadda road followed by Hashtnagri and Karkhano market where three samples were observed positive. In Board bazaar two samples were recorded as positive, where as in Ring road, Haji camp, Gulbahar, Firdos, Sadar, Tahkal and Khyber bazaar one sample was noted positive from each locality.

Salmonella

Salmonella was detected in 4 (6.66%) samples. In Haji camp, Hashtnagri, Charsada road and Karkhano market one sample was observed positive for *Salmonella.*

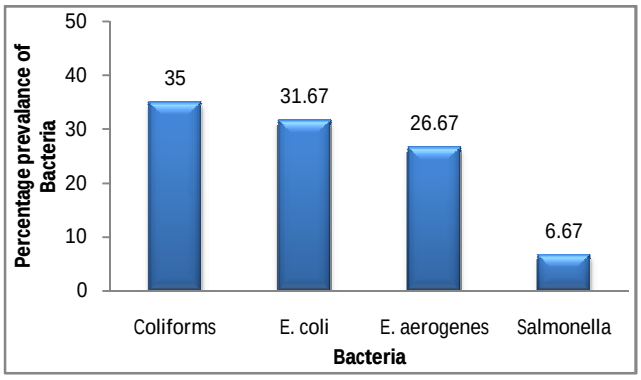

Figure 1: Bacterial prevalence in cows' raw milk.

Table 1: Area wise bacteria isolated from cows' raw milk samples

Sampling area	Total samples	Microorganism isolated			
		Coliform	*E. aerogenes*	*E. coli*	*Salmonella*
Ring Road	5	1	1	1	0
Haji Camp	5	1	1	1	1
Gulbahar	5	1	0	1	0
Hashtnagri	5	3	2	3	1
Firdos	5	1	1	1	0
Charsada road	5	4	2	4	1
Sadar	5	1	1	1	0
Tahkal	5	1	1	1	0
Board Bazar	5	2	2	2	0
Hayatabad	5	1	1	0	0
Khyber bazaar	5	2	2	1	0
Karkhano	5	3	2	3	1
Total	**60**	**21**	**16**	**19**	**4**

Table 2: Biochemical tests results

Gram stain	OX	CT	SH	PAD	VP	MR	Ind	Nit	Mot	Identified bacteria
-ve	-	+	-	-	-	+	+	+	M	*E. coli*
-ve	-	+	-	-	+	-	-	+	M	*E. aerogens*
-ve	-	-	+	-	-	+	-	+	M	*Salmonella*

Biochemical tests

Besides gram staining, various biochemical tests were also performed for identification. These tests included oxidase test (OX), catalase test (CT), phenylalanine deaminase test (PAD), starch hydrolysis (SH), voges-proskauer (VP), methyl red test (MR), nitrate test (NT), indole test (Ind) and motility test (Mot) (Table 2). On the basis of these tests bacteria were identified.

Biochemical (adulterants) analysis

All these sixty samples were analyzed for the presence of chemical adulterants with the help of MAT kit. On all these samples biochemical tests were performed for detection of urea, starch, hydrogen Urea peroxide, ammonium sulphate, boric acid, sugar, salt, carbonates and formalin.

Analysis showed that formalin was present in highest number of samples of 17 with percentage of 28.33% followed by starch which was detected in 16samples with 26.67%. Carbonates were detected in 13samples with 21.67% and sugars were detected in 11samples with 18.33%. Salt and ammonium sulphate both were detected in 10samples with 16.67%. Boric acid was detected in 7samples with 11.67% samples. Urea, hydrogen peroxide and detergents were not detected in any samples.

Starch was detected in sixteen (26.66%) samples (Figure 2). In Firdos, Board bazaar and Khyber bazaar highest no of samples i.e three samples were observed to be adulterated with starch, followed by Ring road and Gulbahar where two samples were noted to be positive for starch. While in Hashtnagri, Sadar and Karkhano market one sample was recorded to be adulterated with starch. Boric acid was detected in seven (11.66%) samples. In Firdos, Charsada road and Board bazaar two samples were noted to be positive from each locality while in Tahkal one sample was noted to be positive for boric acid.

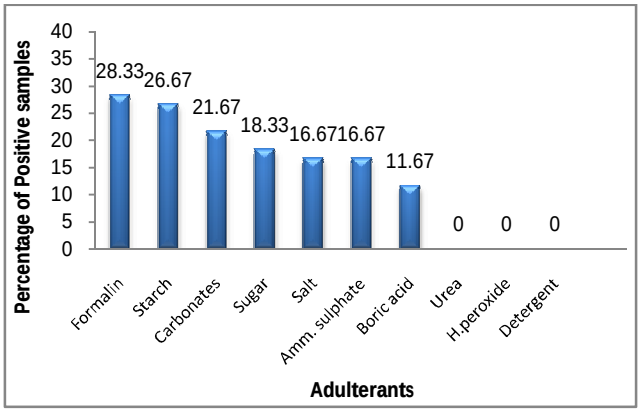

Figure 2: Adulterants profile of raw milk

Sugar detection test was positive for eleven (18.33%) samples. In Tahkal highest number i.e three samples showed the presence of sugar adulteration followed by Haji camp, Sadar and Karkhano where two samples from each locality were positive. Sample obtained from Gulbahar and Charsada was detected positive. Salt test was positive for ten (16.66%) samples. In Firdos one sample was positive, in Ring road, Charsada road and Khyber bazaar two samples were positive while in Hashtnagri three samples were positive for salt test. Carbonate test was positive for thirteen (21.66%) samples. In Haji camp, Gulbahar, Sadar and Khyber bazaar one sample was positive, Ring road, Hashtnagri and Karkhano two samples and in Tahkal three samples were positive for carbonates. Formalin test was positive for seventeen (28.33%) samples. Highest numbers of positive samples for formalin were recorded in Haji camp, Gulbahar and Tahkal where from each locality three samples were positive for formalin. In Ring road, Firdos, Charsada road, Board bazaar, Khyber bazaar and Karkhano one sample was positive, in Sadar two samples. Ammonium sulphate was detected in ten (16.66%) samples. In Haji camp and Hashtnagri highest number i.e three samples were recorded to be positive for ammonium sulphate, followed by Gulbahar where two samples were recorded to be positive for ammonium saulphate. While In Ring road and Charsada road one sample was recorded to be positive.

DISCUSSION

In the present study sixty samples of cows' raw milk were randomly collected from 12 different locations of District Peshawar, including Ring Road, Haji camp, Gulbahar, Hashtnagri, Firdos, Charsada road, Sadar, Tahkal, Board bazaar, Hayatabad, Khyber bazaar and Karkhano market. Five milk samples were collected from each location and were subjected to various tests for detection of bacteria as well as various adulterants.

Being an important food for human, milk also serves as a good source of growth for various microorganisms. Microorganisms that can grow easily in milk include *Lactobacillus, Streptococcus,* Coliform, *Salmonella spp, Staphylococcus* and *Micrococcus spp*. Due to such reason Coliform test including *E.coli* test and *Enterobacter aerogenes* test and *Salmonella* test were performed on collected milk samples. Due to the presence of various types of microorganisms, undesirable changes occur in the milk's appearance, smell and taste. Many microorganisms present in milk, may endanger the consumer's health[18]. Various bacterial species like *Salmonella* are the major and important pathogenic bacteria found in milk[19].

Coliform test were positive for 21 (35%) samples. The detection of coliform and pathogenic bacteria from the milk showed that milk may be polluted from udder of animals, utensils used for getting of milk or the water added in milk[20]. *Enterobacter aerogenes* were present in total 16 (26.67%) samples. Pathogens that have been found to be involved in food borne outbreaks associated with the consumption of milk include *Listeria monocytogenes*, *Escherichia coli, Salmonella spp.,* and *Staphylococcus aureus.* The presence of such types of pathogenic bacteria in milk has caused major public health problems, especially for those individuals who use such contaminated milk[10].

E.coli was detected in 19 (31.66%) samples. These results are in agreement with those of Fook *et al,* who reported that 33.5% samples (312 samples out of 930) were contaminated with *E. coli*[21]. *E.coli* are one of the main leading and important bacteria contaminating the milk[8]. Milk and milk products can be very easily contaminated with *Escherichia coli* and coliform bacteria and their

presence in the milk is a sign of contamination of milk and milk products. The presence of *E. coli* indicates fecal contamination. *E.coli* 0157:H7 has been a serious threat to the dairy industry from many years and outbreaks of different disease have been reported in many countries[22].

Salmonella was detected in 4 (6.66%) samples. The presence of *Salmonella* and such other types of pathogenic bacteria in milk has been the cause for public health problems, especially for those persons who consume *Salmonella* contaminated milk[10]. Jayarao and Henning (2001) reported that worldwide *Salmonella* exist between 2.6 and 25.3% from retail meat, bulk tank milk and fecal samples of dairy cow [23].

The adulteration of food products specially milk is a major problem and may lead to severe health problems. Gastrointestinal problems like gastric ulcer, colon ulcer, diarrhea, and electrolytes disturbance may be caused by carbonates in milk[12]. Hydrogen peroxide adulteration disturbs the antioxidants activity in the body that causes disturbance in natural immunity, which leads to increase aging[13]. Weakening, sensory disturbances and loss of acquired speech may be developed by presence of ammonia in milk. Blood pH and acid base balance in the body may be disturbed by the presence of chlorides in milk[14].

For the analysis of adulteration of milk, Biochemical tests were performed using MAT kit. Urea test, detergent detection tests and Hydrogen peroxide detection tests were negative for all the sixty samples. Reason for absence of these adulterants in market milk samples, may be the ignorance of traders for such practices. Other reason for absence of Hydrogen peroxide adulteration may be that it is not easily and cheaply available in the open market. Our results are in agreement with Rao *et al*, (2002)[24], Karpude *et al*, (1987)[25], Mishra *et al*, (1977)[26] and Patel (1979)[27] who reported that none of the milk samples were adulterated with urea, fertilizers and hydrogen peroxide.

Starch was detected in 16 (26.66%) samples. Sugar detection test was positive for eleven (18.33%) samples. These observations are in agreement with those by Pal (1963)[28], who reported that 0.0 t0 46.3% milk samples in Ludhiana city were positive for sugar.

These results are also in agreement with that of Sanjeevani *et al,* (2011)[29] who reported that in summer season 20% and in rainy season 12% milk samples were adulterated with sugar.

Ammonium sulphate was detected in ten (16.66%) samples. Salt test was also positive for 10 (16.66%) samples. Arora *et al*, (2004)[30] observed that in all collected milk samples from organized and unorganized sector 0.6 per cent samples were positive for salt detection test. In our findings the high presence of salt may be due to easily and cheap availability of salt in open market and awareness of traders regarding such practices.

Boric acid test was positive for seven (11.66%) samples. Formalin test was positive for seventeen (28.33%) samples. Sanjeevani *et al,* (2011)[29] reported that 12% milk sample were positive for formalin. Arora *et al,* (2004)[30] reported that 0.4 per cent milk samples were positive for formalin test. Bansal and Singhal (1991)[31] reported that by addition of formalin in milk even at low concentration; proliferation of any bacteria can be inhibited. High presence of formalin in current study may be due to awareness of milk sellers about these facts that formalin can stop the growth of bacteria in milk and help in keeping quality of milk for longer time. Carbonate test was positive for thirteen (21.66%) samples. This was in agreement with findings of Sanjeevani *et al,* (2011)[29] who reported that 18.00 per cent milk samples in rainy season and 27.00 per cent in winter season were adulterated with carbonates/bicarbonates.

CONCLUSION

In the present study sixty samples of cow's raw milk were randomly collected from 12 different locations of District Peshawar, including Ring Road, Haji camp, Gulbahar, Hashtnagri, Firdos, Charsada road, Sadar, Tahkal, Board bazaar, Hayat abad, Khyber bazaar and Karkhano market. The present study revealed that raw cow milk sold in market is unhygienic due to microbial contamination. An important source of microbial contamination of the milk is faecal pollution probably from cow's dung. It is also concluded that the raw milk samples contained various types of chemical adulterants that may lead to severe health problems. Programs like 'good hygiene practices' and 'good farm practices' should be adopted at every step in milk handling and processing. Moreover, raw milk should not be used without processing (at least boiling) and also strict check and balance system should be developed to control chemical adulteration.

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