Pathogenic bacteria and heavy metals toxicity assessments in evaluating unpasteurized raw milk quality through biochemical tests collected from dairy cows

Hamid Iqbal¹*, Muhammad Ishfaq¹, Muhammad Naseer Abbas², Abdul Wahab², Mughal Qayum², Saifullah Mehsud³

¹Department of Microbiology, Kohat University of Science and Technology, Kohat, Khyber Pakhtunkhwa, Pakistan
²Department of Pharmacy, Kohat University of Science and Technology, Kohat, Khyber Pakhtunkhwa, Pakistan
³Department of Pharmacy, Abbottabad University of Science and Technology, Havelian, Abbottabad, Khyber Pakhtunkhwa, Pakistan

ARTICLE INFO

ABSTRACT

Objective: To evaluate the hygienic quality by determining the presence of predominant pathogenic microbial contaminants (contagious or environmental) and indiscriminate heavy metals contained in unpasteurized milk samples collected from cattle specie of cow.

Methods: Raw milk samples were collected in October, 2014 from different regions of District Kohat, Khyber Pakhtunkhwa, Pakistan and cultured on the selective media plates according to the manufacturer instructions to observe pathogenic microbial flora and confirm it with relevant biochemical tests to specify bacterial specie.

Results: Milk samples analyzed on MacConkey and nutrient agar media were found contaminated mostly with coliform, Staphylococcus aureus, Enterobacter aerogenes and Proteus vulgaris. Similarly, result of the heavy metals analysis performed using atomic absorption spectrophotometer flame photometry showed that raw milk contains heavy metals residues of lead and cadmium contents at higher levels while copper, zinc and chromium were observed lower than permissible limits whereas manganese within specified recommended values.

Conclusions: Microbial contamination of milk and toxic metals is mainly accredited to the scrupulous unhygienic measures during processing of milk exhibiting a wide array of hazardous impacts on human health.

1. Introduction

Milk and milk products are generally regarded as primary source of daily diet containing high quality foods with high biological potential providing both nutritional and culinary values[1]. Human beings and mammalian species feed their infants on milk secretion as it is rich in antibodies and contains significant amount of saturated fats, water, proteins, carbohydrates, minerals, organic acids, enzymes, vitamins and calcium[2]. However susceptibility to spoilage of milk is mainly affected by the presence of microorganisms and toxins, inappropriate handling and storage conditions of temperature, extent of exposure of milk to light and oxygen, maintenance of equipment cleanliness, seasonal changes, soil condition and animal health may vary the contents present in it[3]. Milk in its natural state is found in raw or unpasteurized form that also serves as a medium for the growth of many pathogenic microorganisms like Staphylococcus, Lactobacillus, coliforms, Streptococcus and Micrococcus spp. when consumed. Various zoonotic disorders caused by the presence of bacterial species and verotoxigenic in raw milk samples via production of enterotoxins can cause undulant fever, dysentery, gastroenteritis, food poisoning and intoxication[4]. In addition to pathogenic microbial flora, cattle raw milk also contain important inorganic mineral elements in trace amounts like P, Ca, K, Mg, Na, Cl and trace elements including Cu, Fe, Cr, Cd and Ni. These minerals are required in plants and animals for completion of their life cycles and enzymatic reactions. However, if the toxic level exceed than permissible limits, then it leads to more pronounced health risk factors associated with the consumption or misuse of these heavy metals found contaminated in trace amount as micronutrients in plants[5]. Animals that graze on such contaminated plants and drink from polluted waters accumulate such heavy metals in their vital organs and subsequently take their ways into human...
body through milk secretion mainly by inhalation and ingestion and can cause toxicity. Toxicity of metal in humans and animal health is related to age, route of exposure, frequency and concentration of intake, soil composition, solubility, metal oxidation state, absorption rate, mechanism of excretion, chemical form and pH[6]. A growing body of literature is available in Pakistan on milk adulterants analysis particularly in industrialized and polluted areas as animals graze freely in open fields are considered as bio-indicators of environmental pollution. In view of the growing public awareness about food safety and quality, knowledge of the microbial and heavy metal composition present in milk is of great significance for further development of its hygienic processing into high quality consumer products. The objective of this study was to investigate the occurrence of the opportunistic pathogens of milk-borne infections and heavy metals analysis posed by consumption of raw milk.

2. Materials and methods

2.1. Sampling and media preparations

Fresh unprocessed raw milk samples approximately 5 ml were collected manually from cow and inoculated into peptone broth. After incubation at 37 °C for 18–24 h, 100 μL of the inoculated broth was streaked onto Petri plates containing MacConkey and nutrient agar media and incubated again at 37 °C for overnight. Microorganisms developed on the plates were sub cultured and analyzed for Gram staining reaction as it is one of the first procedure used to classify bacteria observed under microscope. Coliform count was performed by plating milk sample on violet red bile agar, a media that selects for coliform bacteria. Similarly eosin methylene blue (10 mL) manufactured by Oxoid (CM0003) was poured onto Petri plates for suspected colonies of Escherichia coli (E. coli) and Enterobacter aerogenes (E. aerogenes). Suspected colonies of Salmonella were streaked and purified on selective medium Salmonella-Shigella agar (10 mL), autoclaved for 20 min at 121 °C and was poured into sterile Petri plates and incubated for 48 h at 37 °C. Salmonella produced colorless to pale pink or blue opaque, transparent or translucent colonies. There are several methods available for detection and enumeration of microorganisms in raw milk. All the experiments conducted were further identified using biochemical tests in duplicate to evaluate the hygienic quality of milk[7].

2.2. Microbial analysis of microorganisms

Following is a summary of raw milk quality parameters, testing procedures with desirable permissible standards.

2.3. Direct microscopic examination

Raw milk sample was spread on microscopic slide using a platinum wire loop to apply the milk resulting in a smear of one square centimeter area. After drying, xylol was flooded on slide then stained with methylene blue solution and examined under light power (40×) microscope and finally moved to oil immersion lens at 100×. The plated samples were allowed to cool before inverting and placing them in the incubator at 37 °C for 48 h to quantify the presence of microorganisms.

2.4. Raw milk quality tests/biochemical tests

Confirmation of these isolated bacteria was conducted with classical biochemical tests for each experiment carried out in duplicate with appropriate positive and negative controls as described below in Table 1.

| Gram stain | Oxidase test | Catalase test | Starch hydrolysis test | Phenylalanine deaminase test | Voges-proskauer test | Indole test | Nitrate reductase test | Motility test | Identified bacteria |
|------------|--------------|---------------|------------------------|-----------------------------|----------------------|------------|------------------------|--------------|---------------------|
| -          | -            | +             | -                      | -                           | +                    | -          | +                      | Motile       | E. aerogenes        |
| -          | -            | +             | -                      | -                           | -                    | +          | +                      | Motile       | E. coli             |
| -          | -            | -             | +                      | -                           | -                    | -          | +                      | Motile       | Salmonella          |

+: Positive; -: Negative.
Voges-Proskauer test was performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth to detect the presence of acetoin, a precursor of 2, 3 butanediol. A cherry red color indicates a positive result for acetoin while a yellow-brown color indicates a negative result.

Indole test was conducted for the identification of organisms having tryptophanase enzymes which convert amino acid tryptophan into indole. The organism was inoculated in a test tube containing culture broth with the addition of 0.5 mL of Kovac’s reagent (isosamyl alcohol, para-dimethylaminobenzaldehyde + hydrochloric acid) and incubated at 37 °C for 24 h. Development of red-violet ring layer indicates positive whereas yellow ring layer indicates negative test.

Nitrate reductase test was used to differentiate between microbes based on their ability or inability to reduce NO₃ to NO₂ using anaerobic respiration. An inoculum aseptically transferred to a sterile inverted Durham tube of nitrate broth was incubated at 35–37 °C for 24 h. A positive test for both enzymes (nitrate reductase and nitrite reductase) consists of a turbid (cloudy) broth with pronounced gas bubbles trapped in the Durham tube, indicated by the broth turning red.

Motility test was used to determine the ability of an organism to swim randomly with flagella monitored under microscope. Turbidity in the entire tube against the current of water streaming indicates that bacteria are motile in a wet mount and vice versa.

2.5. Heavy metals analysis

2.5.1. Acid digestion of milk

All glass wares were washed with 10% HNO₃ solution and distilled water. A milk quantity of 20 mL was digested with 1:3 of H₂O₂ and HNO₃ solution respectively. Raw milk samples were heated on a hot plate until volume reduced to 5 mL and then diluted with 20 mL distilled water. Analysis of the heavy metals in the milk samples was performed using atomic absorption spectrophotometer with 20 mL distilled water. Analysis of the heavy metals in the milk samples was performed using atomic absorption spectrophotometer with suitable hollow cathode lamps and the observed values were compared with the Perkin-Elmer 3110 model. Analysis of the heavy metals in the milk samples was performed using atomic absorption spectrophotometer with suitable hollow cathode lamps and the observed values were compared with the Perkin-Elmer 3110 model. Analysis of the heavy metals in the milk samples was performed using atomic absorption spectrophotometer with suitable hollow cathode lamps and the observed values were compared with the Perkin-Elmer 3110 model.

Motility test was used to determine the ability of an organism to swim randomly with flagella monitored under microscope. Turbidity in the entire tube against the current of water streaming indicates that bacteria are motile in a wet mount and vice versa.

| Heavy metals analysis | Mean mg/kg | Permissible limit mg/kg | Toxic limit mg/kg | Daily dietary intake |
|-----------------------|------------|-------------------------|------------------|---------------------|
| Lead                  | 1.720      | 0.026                   | > 500 μg/L       | 20–280 μg/day for adults |
| Cadmium               | 0.461      | 0.049                   | 200 μg/kg for infants | 10–275 μg/day for adults |
| Copper                | 0.141      | 0.400                   | 3 μg/kg          | 2–5 μg/day for children |
| Zinc                  | 3.136      | 4.800–5.400             | 150 μg/kg        | 3000 μg/day          |
| Manganese             | 0.056      | 0.025                   | 15 μg/kg         | 2–5 μg/day for adults |
| Chromium              | 0.041      | 0.500                   | 2.5–25.0 μg/kg   | 50–200 μg/day        |

Table 2 shows the quantitative determination of heavy metals concentration to assess the residues levels as mean value (mg/kg) examined in unpasteurized raw milk samples collected and evaluate the potential health risks of metals to humans via consumption of milk and dairy products. Investigational study conducted also shows range of the heavy metals (lead, cadmium, copper, manganese and chromium), permissible limit, toxic limit and daily diary intake values.

![Figure 1](image)

**Figure 1.** Pictures show the growth of bacteriological analysis observed on Petri plates under different growth conditions and incubation periods on selective media preparations as follows.

a: Growth of *E. aerogenes* on eosin methylene blue; b: Growth of *E. coli* on eosin methylene blue; c: Growth of *Salmonella* on Salmonella-Shigella agar; d: Growth of coli forms on violet red bile agar.

3. Results

The results of the present study for all the positive samples for bacterial identification were based on growth of bacteria on selective agar and broth, colony morphology, Gram’s reaction, biochemical test results (Table 1) and criteria for disregarding negative cultures. This study indicated that the dominant microbial flora associated in raw milk samples were *E. aerogenes*, *E. coli*, *Salmonella* and faecal coli forms as shown in Figure 1.

4. Discussion

Recently, it has been observed that the ground soil quality is getting drastically polluted and have resulted in ultimate disposal of microbial counts and toxic metals. Subsequently, these are taken by animals including human beings and take their ways into milk and milk products during production and processing of milk rendering serious threats to human health. Being an important food for human, milk also serves as a medium of growth for various microorganisms like *Lactobacillus* spp., *Streptococcus* spp., coli form, *Salmonella*.
spp., Staphylococcus spp. and Micrococcus spp.[9]. The presence of these predominant microorganisms has been abundantly reported in unpasteurized milk samples that may endanger the consumer’s health and lead to food borne outbreaks[10]. Keeping in view the importance of health hazards, actual and indirect biochemical tests given in Table 1 were performed for each case.

The microscopic examination of raw milk is often used for high bacteria counts based on color differences (pink color shows Gram-negative organisms while purple indicates Gram-positive) and morphology (shape, color and size). While milk smears may provide evidence of microorganisms associated with poor cooling and mastitis, other causes of high counts may not be as representative. In many cases, further characterization is required. Culture techniques are the most common but along with failure to isolate viable non-culturable organisms it is also time consuming to produce results. On the other hand, as discussed by Doyle et al.[11], total direct microscopic count methods are relatively fast, but limitations of these techniques can lead to numerous errors or misdiagnosed of the organisms like (a): failure of all organisms to grow at specified media and incubation temperatures used; (b): single colonies developing from clumps of organisms; (c): sampling and technique errors; (d): factors involve in lowering direct count; (e): failure of all organisms to stain, or staining of dead organisms; (f): inability to discriminate between living and dead bacteria[12]. The detection of bacterial counts is revealed by the presence of pathogenic bacteria such as E. aerogenes (Figure 1a), E. coli (Figure 1b), Salmonella (Figure 1c) and coli forms (Figure 1d). Environmental determinants causing Enterobacteriaceae is probably the result of fecal micro flora representing an obvious risk of damage to the mammary glands of farm animals called mastitis. Moreover, the presence of pathogens such as E. coli also reflects the health status of the animal living in unhygienic practices. Salmonella infections are commonly found in regions with intensive animal husbandry and subjected to fecal contamination. Food borne gastroenteritis have been attributable to raw, undercooked or contaminated dairy products[11]. Presumptive test for the presence of coliform was positive in all milk samples. Appearance of dark red colonies indicated the presence of coliform. Coliforms because of their presence in food indicate some form of fecal and filth contamination (e.g., dirty cows and dirty equipment). It is also indicated that hygienic counts of less than 50/mL is linked with total number of bacteria per milliliter.

Similar to the conductance of our study, Mubarak et al.[13] investigated the microbiological quality of raw milk as unhygienic and revealed a definite dominance of Lactobacillus spp. along with other genera of Escherichia, Bacillus, Salmonella and Pseudomonas isolated on selective agar and broth media. A study by Ekici et al.[14] on raw milk samples of sheep, goat and cow and revealed the presence of Staphylococcus aureus and E. coli with the exception of Salmonella spp. in any of the samples. Microbial analysis of raw milk samples observed in different regions of Dehradun (India) revealed a dominant micro flora of E. coli, Micrococcus, Lactobacillus spp, Salmonella spp, Staphylococcus aureus and Klebsiella species among the isolates[12].

Growing tendency and increase in demand for food has drawn the attention of researchers to the health risk factors associated with consumption of heavy metals that are found contaminated in food stuffs and dairy products too. Calculated observed values as mean are discussed as below.

Pb²⁺ is one of the most noxious non essential heavy metal implicated in causing carcinogenesis, hematopoietic, renal and gastrointestinal disturbances. Lead concentration was observed in cow milk sample as 1.72 mg/kg (Table 2) while permissible limit set according to Marina et al.[15] is 0.026 mg/L. In blood, normal level of Pb is below 400 µg/mL. Our study showed much higher results with findings of Lawal et al.[16] as (0.531 ± 0.2990) mg/L, Semaghuil et al.[17] as 0.12 mg/L and similar observations set with Abdallah[18] as (2.55 ± 0.12) mg/kg.

Cd²⁺, being a highly toxic pollutant of soil, inhibits crop production, affects nutrient uptake and has significant potential to impair animal and human health (it is implicated in high blood pressure, prostate cancer disorders. The calculated Cd concentration (0.461 mg/kg) was found higher (Table 2) compared to recommended values. Our findings are in contrast with those reported by Lawal et al.[16] as (0.0257 ± 0.1270) mg/L, Semaghuil et al.[17] as 0.004 mg/L and similar to the observations by Abdallah[18] as (0.41 ± 0.05) mg/kg.

Cu⁺ is mainly absorbed in liver and bone marrow. High concentration of copper leads to vomiting, diarrhea and cardiovascular collapse. Copper concentration was found in the milk sample as 0.141 mg/kg as shown in Table 2. The provisional tolerable daily intake for copper is 3 mg[19] while permissible limit set according to[20] is 0.4 mg/kg. The low concentrations of Cu could be due to Zn contained in food that interferes with the copper absorption system. Similar results were observed by Lawal et al.[16] as (0.062 ± 0.026) mg/L, Semaghuil et al.[17] as 0.17 mg/L and (0.110 ± 0.002) mg/kg by Abdallah[18].

Zn⁺⁺ concentration was found in the milk of cow as 3.136 mg/kg (Table 2). All the values in the study samples were below the permissible limit (4.8–5.4 mg/kg). Chronic zinc exposure results in blood disorders and gastrointestinal diseases. Our results were similar with findings of Abdallah[18] as (3.661 ± 0.003) mg/kg and in contrast with results of Semaghuil et al.[17] as 0.98 mg/L.

Mn⁺⁺ is an essential nutrient for normal physiological functions with minimal adverse effects such as impaired neurological and neuromuscular disorders at higher doses[21]. Mn concentration in milk sample was found as 0.056 mg/kg shown in Table 2. The daily dietary intake is 2–5 mg/day for adults and 2.5–25.0 µg/kg body weight for infants[22]. Similar observation (0.08 mg/L) was recorded by Semaghuil et al.[17].

Cr⁺⁺ is an essential element as it uptakes sugar, protein and fat. In addition it also maintains blood cholesterol level and stimulates the activities of insulin regulated inside the body. Chromium is one of the major discharges in tannery waste. Excessive amount of Cr leads to carcinogenesis. Cr concentration in cow was found as 0.041 mg/kg (Table 2). The permissible limit for Cr in food is generally
0.5 mg/L while acceptable daily intake of chromium is 50–200 μg/day[23].

It is clear from investigation that presence of the pathogenic organisms, coliform counts and heavy metals are indicative of potentially hazardous products, posing a serious threat to the consumers. This may be attributed to favorable conditions like ambient temperature required for the growth of bacteria, unhealthy and unsanitary practices mainly by illiteracy factors and significant environmental pollution. The hygienic quality of milk is of crucial importance in producing milk and milk products that are safe and suitable for their intended uses. Milk contamination can be decreased by proper hygienic packaging of milk, standard pasteurization and refrigerated temperature employed for the destruction of pathogens at recommended level. Concerned authorities and health regulatory departments including veterinarians and dairy farmers should ensure regular monitoring and effective control measures for hygienic production and good nutritive quality of milk for drinking purposes and dairy products.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are grateful and wish to express thank to the Chairman Department of Microbiology, Kohat University of Science and Technology and Chairman Department of Chemistry, Kohat University of Science and Technology and all the faculty members for gratitude and making the facilities available for the research.

References

[1] Khan I, Zeb A. Nutritional composition of Pakistani wheat varieties. J Zhejiang Univ Sci B 2007; 8: 555-9.
[2] Torkar KG, Teger SG. The Microbiological quality of raw milk after introducing the two day’s milk collecting system. Acta Agric Slovenica 2008; 92: 61-74.
[3] Khattak B, Iqbal H, Abdul QK, Sherwani SK, Shah MA, Khan AQ. Microbial analysis and quality control of milk collected from various districts of Khyber Pakhtunkhwa. Int J Pharm Res Bio-Sci 2013; 2: 243-52.
[4] Bielmann V, Gillan J, Perkins NR, Skidmore AL, Godden S, Leslie KE. An evaluation of Brix refractometry instruments for measurement of colostrum quality in dairy cattle. J Dairy Sci 2010; 93: 3713-21.
[5] Iqbal H, Khattak B, Ayaz S, Rehman A, Ishaq M, Abbas MN, et al. Pollution based study of heavy metals in medicinal plants Aloe vera and Tamarix aphylla. J Appl Pharm Sci 2013; 3(4): 54-8.
[6] Aslam B, Javed I, Khan FH, Zia-ur-Rahman. Uptake of heavy metal residues from sewerage sludge in the milk of goat and cattle during summer season. Pak Vet J 2011; 31: 75-7.
[7] Wehr HW, Frank JF. Standard methods for the examination of dairy products. 17th ed. Washington D.C: American Public Health Association; 2004, p. 34-6.
[8] Bilandzic N, Dokie M, Sedak M, Solomun B, Varenina I, Knezevic Z, et al. Trace element levels in raw milk from northern and southern regions of Croatia. Food Chem 2011; 127(1): 63-6.
[9] Muma JB, Pandey GS, Munyeme M, Mumba C, Mkandawire E, Chimana HM. Brucellosis among smallholder cattle farmers in Zambia: public health significance. Trop Anim Health Prod 2012; 44: 915-20.
[10] Ndungu TW, Muliro PS, Omwamba M, Oosterwijk G, Jansen A. Quality control of raw milk in the smallholder collection and bulking enterprises in Nakuru and Nyandarua Counties, Kenya. Afr J Food Sci 2016; 10: 70-8.
[11] Doyle MM, Garcia S, Bahati E, Karamuzi D, Cullor JS, Nandi S. Microbiological analysis of raw milk in Rwanda. Afr J Food Sci Technol 2015; 6: 141-3.
[12] Pant R, Nirwal S, Rai N. Prevalence of antibiotic resistant bacteria and analysis of microbial quality of raw milk samples collected from different regions of Dehradun. Int J PharmTech Res 2013; 5: 804-10.
[13] Mubarak MH, Doss A, Dhanabalan R, Bakachandar S. Microbial quality of raw milk samples collected from different villages of Coimbatore District, Tamilnadu, South India. Indian J Sci Technol 2010; 3: 61-3.
[14] Ekici K, Bozkurt H, Islyci O. Isolation of some pathogens from raw milk of different milch animals. Pak J Nutr 2004; 3(3): 161-2.
[15] Campos MM, Tonucci H, Silva SM, de S Altoe B, de Carvalho D, Kronka EA, et al. Determination of lead content in medicinal plants by pre concentration flow injection analysis-flame atomic absorption spectrometry. Phytochem Anal 2009; 20: 445-9.
[16] Cai Q, Long ML, Zhu M, Zhou QZ, Zhang L, Liu J. Food chain transfer of cadmium and lead to cattle in a lead-zinc smelter in Guizhou, China. Environ Pollut 2009; 157(11): 3078-82.
[17] Birghila S, Dobrinas S, Stanciu C, Soceanu A. Determination of major and minor elements in milk through ICP-AES. Environ Eng Manage 2008; 6: 805-8.
[18] Gonzalez-Montana JR, Senis E, Gutierrez A, Prieto F. Cadmium and lead in bovine milk in the mining area of the Caudal River (Spain). Environ Monit Assess 2012; 184(7): 4029-34.
[19] Ogabiela EE, Yebpella GG, Ade-Ajayi AF, Mmereole UJ, Ezeanyasok C, Okonkwo EM, et al. Determination of the level of some elements in edible oils sold in zaria, northern Nigeria. Global J Pure Appl Sci 2010; 6: 325-31.
[20] Blanco-Penedo I, Cruz JM, Lopez-Alonso M, Miranda M, Castillo C, Hernandez J, et al. Influence of copper status on the accumulation of toxic and essential metals in cattle. Environ Int 2006; 32(7): 901-6.
[21] TEACH. Toxicity and exposure assessment for children’s health. Chicago: TEACH; 2007. [Online] Available from: https://archive.epa.gov/region5/teach/web/html/index.html [Accessed on 13th August, 2016]
[22] Perveen F, Mumtaz M, Usmani TH. Estimation of metal contents in different varieties of milk available in Karachi city. J Chem Soc Pak 2005; 27(6): 611-4.
[23] Lopez Alonso M, Prieto Montaña F, Miranda M, Castillo C, Hernandez J, Luis Benedito J. Interactions between toxic (As, Cd, Hg and Pb) and nutritional essential (Ca, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn) elements in the tissues of cattle from NW Spain. Biometals 2004; 17(4): 389-97.