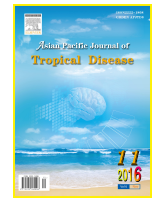




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Pathogenic bacteria and heavy metals toxicity assessments in evaluating unpasteurized raw milk quality through biochemical tests collected from dairy cows

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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

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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.

Oxidase test was used to distinguish between oxidase positive *Pseudomonadaceae* and oxidase negative Enterobacteriaceae microorganisms containing the enzyme cytochrome oxidase (a hemoprotein) which transfers electrons from the electron transport chain to oxygen (the final electron acceptor) and reduces it to water. When the electron donor is oxidized by cytochrome oxidase, it turns a dark purple and is indicative of a positive result.

Catalase test was used to identify organisms that produce the enzyme, catalase and detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. Production of oxygen gas with bubbles indicates positive result.

Starch hydrolysis test was used to differentiate species from the genera *Bacillus* and *E. coli* using the enzymes alpha amylase and oligo-1, 6-glucosidase. After inoculation and overnight incubation at 37 °C, iodine reagent forms complexes in the presence of starch. Appearance of clear halos surrounding colonies is indicative of their ability to digest the starch in the medium due to the presence of alpha-amylase. This is a negative reaction for the starch hydrolysis test.

Phenylalanine deaminase medium tests the ability of an organism to produce the enzyme deaminase and was used to differentiate members of the genera *Proteus*, *Morganella* and Enterobacteriaceae. After incubation, 10% ferric chloride was added to the media. If phenylpyruvic acid was produced, it will react with the ferric chloride and turn dark green. If the medium remains a straw color, the organism is negative for phenylalanine deaminase production.

Table 1

Biochemical tests.

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	<i>E. aerogenes</i>
-	-	+	-	-	-	+	+	Motile	<i>E. coli</i>
-	-	-	+	-	-	-	+	Motile	<i>Salmonella</i>

+: 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 (isoamyl 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 NO_3 to NO_2 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% HNO_3 solution and distilled water. A milk quantity of 20 mL was digested with 1:3 of H_2O_2 and HNO_3 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 flame photometry (Perkin-Elmer 3110 model) with suitable hollow cathode lamps and the observed values were compared with the standard as stated by the Food and Agriculture Organization/World Health Organization Joint Expert Committee on Food Additives[8]. Each step was carried out in duplicate in the laboratory of Chemistry, Department of Kohat University of Science and Technology, Kohat, Pakistan.

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.

Table 2

The Quantitative determination of heavy metals concentration (mg/kg).

Heavy metals analysis	Mean mg/kg	Permissible limit mg/kg	Toxic limit	Daily dietary intake
Lead	1.720	0.026	> 500 $\mu\text{g/L}$	20–280 $\mu\text{g/day}$ for adults 10–275 $\mu\text{g/day}$ for children
Cadmium	0.461	0.049	200 $\mu\text{g/kg}$ for infants	15–50 $\mu\text{g/day}$ for adults 2–25 $\mu\text{g/day}$ for children
Copper	0.141	0.400		3000 $\mu\text{g/day}$
Zinc	3.136	4.800–5.400	150 $\mu\text{g/day}$	15 $\mu\text{g/day}$ or 60 mg/L for adults
Manganese	0.056	0.025		2–5 mg/day for adults 2.5–25.0 $\mu\text{g/kg}$ for infants
Chromium	0.041	0.500		50–200 $\mu\text{g/day}$

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, zinc, manganese and chromium), permissible limit, toxic limit and daily dietary intake values.

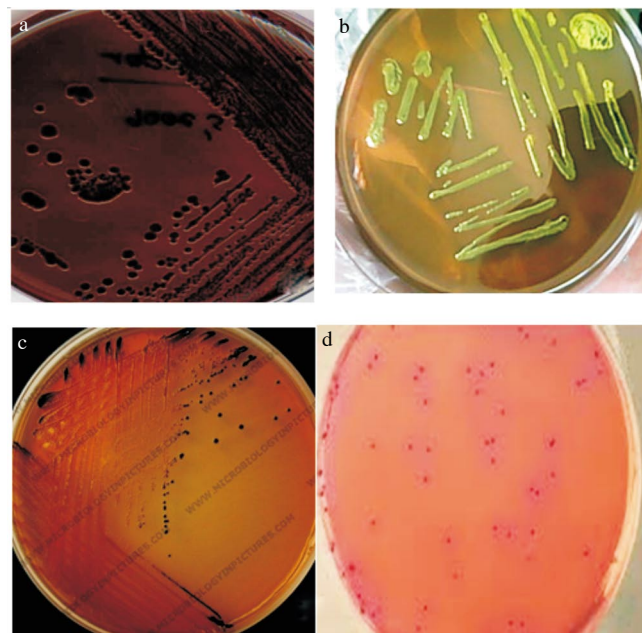


Figure 1. Pictures show the growth of bacteriological analysis observed on Petri plates under different growth conditions and incubation periods on selective media preparations as follows.

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.

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 (0.5312 ± 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.

Cu¹⁺ 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].

Zn¹⁺ 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.

Mn²⁺ 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].

Cr³⁺ 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.

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