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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 ℃ 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 ℃ 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 ℃ and was poured into sterile Petri plates and incubated for 48 h at 37 ℃. *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 ℃ 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 ℃, 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	*E. aerogenes*
-	-	+	-	-	-	+	+	Motile	*E. coli*
-	-	-	+	-	-	-	+	Motile	*Salmonella*

+: Positive; -: Negative.