DETECTION OF *MYCOBACTERIUM* FROM BOVINE MILK IN LAHORE, PAKISTAN. (Research Report)

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ABSTRACT: It is reported since 100 years that milk is a source of contamination for the spread and transmission of Tuberculosis from animals to human beings and vice versa. To validate this hypothesis, current study was designed to record the prevalence of Mycobacterium spp. from the bovine milk and their chances of transmission of disease. Heat treated and untreated bovine milk samples (n=210) were obtained from different areas of Lahore including Wall city, Zainbia Multan road, Itaifaq town, Shahdara, Iqbal town, Jail road from January to June 2012. 4.28% prevalence of Mycobacteria spp. was observed using Ziehl-Neelsen staining, microscopic and culturing techniques. The study showed that the untreated milk consumption poses a threat of transmission of Mycobacteria from one animal to another and even to human beings.

KEY WORDS: Mycobacterium, Bovine, Milk.

INTRODUCTION

Milk is the most important part of the human diet which is used in different forms as food. It plays prominent role in meeting the essential human dietary requirements. Approximately 50% of the milk produced from the dairy animals is consumed as fresh, boiled or in pasteurized form. However one sixth of this is consumed as yogurt or curd and remaining is utilized for the manufacturing of indigenous varieties of milk products such as butter, khoya, paneer rabri, kheer, barfi, gulabjamin and ice-cream etc [1]. In Pakistan, during the year 2010-2011, livestock contributed nearly 55.1 % of the agricultural value and 11.5 % of the National GDP [2]. The major population of buffalo is present in the province Punjab, Pakistan and is known as milch of Pakistan. Buffalo is also referred as black gold of the country [3]. Pakistan stands at 2nd position with approximately 27.3 million herds of buffaloes after India with 98 million and before China with 23 million herds of buffaloes [4]. This contributes approximately 67% of the total milk production in the country. The economy of the country is directly linked with the livestock and its importance can be gauged from the fact that approximately 30-35 million rural people are engaged in the raising of livestock. These families generate 30-40% of the income from these animals [3].

Tuberculosis (TB) is the major contributor to the global burden of disease and is especially present in the countries which are in developing state [5]. Tuberculosis is the specific infectious chronic disease, caused by the *Mycobacterium* species. The tuberculosis primarily affects the lungs and cause pulmonary tuberculosis but the secondary phase of tuberculosis infects the intestines, meninges, bones, joints, lymph glands, skin, kidneys and other tissues of the body [6]. The animals of bovine family are affected with *tuberculosis* due to the *Mycobacterium bovis*. *Mycobacterium bovis* also produces ailment in human beings as well as in animals. It is transmitted form animals to humans through unpasteurized milk and its by-products, sputum, urine, visceral organs, nasal discharges or aerosols [7]. Bovine tuberculosis is a disease of high economic relevance within the context of livestock farming because it directly affects the productivity of animal and also influences the trade of the animal products.

Among the infectious diseases, TB is considered to be the second most common disease around the world and killing almost 2 million people annually. According to the report in 1999 around 8 million new cases of TB are recorded annually, thereby representing a major economic burden on individuals and countries [8]. World Health Organization ranked Pakistan 6th in world in terms of tuberculosis burden [9]. According to the report of World Health Organization in 2009 the highest incidence rate of TB was reported in Sub-Sahara Africa while Bangladesh, Pakistan, India, China and Indonesia together account for half of the TB burden round the globe. Pakistan has been reported as one of the twenty two countries accounting for the total TB burden worldwide and is categorized in one of the five countries responsible for the tuberculosis worldwide [10]. In Pakistan, bovine tuberculosis was a cause of threat for animals and humans in both the public and private sector [11]. Recently the prevalence of disease has been reported to be 7.6% in cattle (11 different stations) and 3% in buffaloes (two cities) respectively [12, 13].

There is a strong link, cross adaptability and transmission of *Mycobacterium* between the animal and human tuberculosis [14]. The bovine tuberculosis is detected by the delayed hypersensitivity test in live animals and from the post-mortem examination in dead animals. The presence of *Mycobacterium* in apparently healthy animals is diagnosed by cultural examination or by using modern molecular diagnostic techniques. Bacteriological examination includes demonstration of acid-fast bacilli by microscopic examination, isolation and identification of the *Mycobacterium* on their selective media and biochemical tests.

Bovine tuberculosis is an important zoonotic problem worldwide, which has no known geographical boundaries and can infect the groups of animals having great economic importance in wildlife and human. *M. bovis* is the member of the *M. tuberculosis* complex, a group that also includes *M. tuberculosis, M. africapum and M. microti*. *M. bovis* the

causative agent of bovine tuberculosis and *M. tuberculosis*, the causative agent of tuberculosis in humans are genetically and antigenically very similar and cause identical clinical disease in humans [15]. It is estimated that animal affected with tuberculosis lose 10-25% of their productive efficiency which is apart from the mortality rate of animal [16].

Mycobacterium is slender, slightly curved rod shaped bacteria. It has a complex peptidoglycan- arabinogalactan mycolate cell wall that is approximately 60% lipid. It stains poor with Gram stain but a highly cross-linked peptidoglycan and no endotoxin. It is an acid-fast bacillus and retains carbol fuchsin even when it is decolorized by acid alcohol (because of the presence of the long chain fatty acids called mycolic acids in the cell wall). It is resistant to acids and alkali which are used for the treatment of sputum to reduce normal contamination of bacteria before culturing Mycobacteria. Due to the presence of the single copies of ribosomal genes *Mycobacteria* are slow grower. It is resistant to drying and many disinfectants. In healthy hosts it stimulates a strong cell-mediated immune response [17].

Bovine tuberculosis is a chronic disease of animals particularly in buffaloes associated with the progressive weakness, disorder of respiratory systems primarily in the lungs which then with the passage of time pass to other organs [18]. The transmission of *Mycobacterium* from animal to animal occur through colostrums/milk to calves, by the ingestion of the infected flies, by the dropping of birds, aerosols, contact with one and the other animals [19]. The animals infected with bovine tuberculosis secrete a causative organism i.e., the *Mycobacterium bovis* in its milk and nasal secretions. Due to the transmission of *Mycobacteria* from animals to humans, this disease has significant importance. The chances of the infection of *Mycobacteria* will increase if the humans use the unpasteurized or not properly treated milk. That's why bovine tuberculosis is emerging as the important extra-pulmonary human tuberculosis [20]. Transmission of *M. bovis* can occur between the animals, from animals to humans and also through humans to animals, but rare from human to human.

Some investigations have pointed out the risk of human infection through unpasteurized, untreated consumption of milk or using raw milk for producing cream, butter or dahi (curd) among cattle owners and herdsman in community [21]. *Mycobacterium bovis* can also transfer from humans to animals. During grazing the animal may become the victim of this disease by ingesting the *Mycobacterium bovis* within its body [11].

OBJECTIVE:

The objective of this study was to find out the presence of *Mycobacterium* in the raw or unpasteurized milk and its chances of transmission to human beings and other animals. Moreover the comparison was made among different milk packing like heat treated samples (tetra-packs and pasteurized milk packet) and heat untreated milk samples (raw milk and direct milk at the time of milking).

MATERIALS AND METHODS

The study was designed to determine the prevalence of *Mycobacteria spp*. in milk samples from different areas of Lahore, Pakistan. Two hundred and ten (210) milk samples were collected from January 2012 to June 2012, from different sources including the tetra-packs, pasteurized milk packets, direct milk at the time of milking from the herds and from the common milk shops of Waal city, Shahdara, Itefat town, Iqbal town, Jail road and Zainbia Multan road Lahore.

The milk samples (5ml) were collected from different sources in sterile containers and stored at 4°C till further use. The samples under refrigeration were transported to the Bacteriology Department of Institute of Public Health (IPH) for staining, culturing and identification of *Mycobacterium* by microscopic examination. The milk samples of different packs were collected as: Tetra-packs 25 packets, Pasteurized milk packets 33 packets, Milk shops 92 samples and Common herd 60 samples.

Tetra-packs: Tetra-packs of different batches all the companies i.e., Milk pack, Haleeb, Olper's, Dairy pure, Tarang, Dairy Umang, Nestle everyday (liquid), Chaika were taken and examined. Total twenty five tetra-packets of different batches were collected at different time intervals.

Pasteurized milk packets: pasteurized milk packets including the milk packets of Gourmet, Doce, Hala and Nurpur and from the bakeries were collected and observed. Total thirty three pasteurized milk packets were collected and studied.

Milk Shops: Milk from the common milk shops were obtained from the Waal city, Mansoora and its nearby areas of Lahore, Pakistan. Total ninety two samples from different shops were collected and processed on the given time interval.

Common herd: Milk from the common herd means the milk obtained at the time of milking. Last streams of milk were collected in the sterile containers and were stored at 4° C till the further use. Total 60 milk samples were collected and studied.

Preparation of milk samples

Milk samples were collected in sterile containers. These samples were stored at 4°C till further use. After that 5ml milk was taken in Falcon tube. The milk samples were centrifuged at 3000 rpm for 15 minutes and the super-natant was discarded. The sediments were suspended in 2ml of sterilized physiological saline solution. To the suspension was added equal volume of sterilized 4N sodium hydroxide solution and one drop of 0.05% phenol red indicator and the mixture was incubated for 30 minutes at 37°C. Finally the samples were neutralized with 4N hydrochloric acid and were centrifuged at 3000 rpm for 15 minutes, and sediment was used for the microscopic and cultural examination [22].

Culture Method

Milk samples of the animals were cultured on Lowenstein-Jensen medium with the addition of glycerol for *Mycobacterium tuberculosis* and without the addition of glycerol (add sodium pyruvate in place of glycerol) for *Mycobacterium bovis*. Thick inoculums of the sediments were smeared on the surface of the medium slopes and the culture tubes were incubated at 37°C for 6 to 8 weeks [23]. Using aseptic technique, dissolved the dye in sterile distilled water. Placed the mixture in incubator at 37° C for 1-2 hrs or

heat in water bath at 37° C. Stored in dark bottles. This solution was not stable long-term; if precipitation occurs, discard and prepared fresh solution.

Prepared salt solution by dissolving components in distilled water. Autoclaved it at 121° C for 30 minutes in screw-capped bottles. Because the structure of T.B bacteria is different from other bacteria that's why to recognize it we use special type of staining technique known as Ziehl Neelsen staining method and then examined the slides under oil-immersion lens [24]. Covered the slide with carbol fuchsin it colorized all the ingredients in smear. Decolorized it with 25% sulfuric acid. It decolorized all the components in the smear except the T.B bacteria. The T.B bacteria absorbed this red color and known as Acid Fast Bacilli (AFB). Now cover the smear with methylene blue. All the components developed blue color due to this that's why the smear appears blue in color.

Microscopic Examination:

The stained slides which were observed under oil-immersion lens to find out the *Mycobacteria*. The *Mycobacteria* appeared red in color with blue background due to methylene blue.

RESULTS:

Heat treated and untreated milk samples (n=210) were collected from January 2012 to June 2012 from different sources. The heat treated milk samples included tetra-packs, pasteurized milk packets and heat untreated milk samples included from common milk shops and herds. Herds included the last streams of the milk at the time of milking. The milk samples were collected from Wall city, Shahdara, Zainbia Multan road, Itaifaq town, Iqbal town and Jail road Lahore. Out of 210 milk samples 9 milk samples were positive for *Mycobacteria*. The prevalence of the disease during this time period was 4.28%.

During six months course of study, collectively two hundred and ten milk samples were collected. In January only one milk sample was found positive while in February the point prevalence was zero because no sample was found positive. March 2012 gave one positive sample. The prevalence of *Mycobacteria spp*. in the month of April and June was 5.71% as two samples were found positive. The maximum positive samples were observed in the month of May this is because in these months the temperature was ideal for the growth of *Mycobacteria*. Overall results showed 9 positive samples which showed overall 4.28% incidence. During the month of January total thirty five samples were observed for the presence of *Mycobacteria*. Out of these thirty five samples four tetra-pack milk samples, five pasteurized milk packets, seventeen samples from milk shops and eight herd samples were observed. Only one sample was found positive for the *Mycobacterium*, out of 35 samples. During the month of February total thirty five samples not a single sample was found positive. This may be because of the reason that low temperature did not support the growth *of Mycobacteria* in the milk samples. The point prevalence was zero as no single sample was positive.

During the time period of the study in the March 2012, overall only one milk sample was found positive and left 34 samples were negative for *Mycobacteria*. The point prevalence was 8.3%. Only one sample from the twelve herds was found positive and all the milk samples of tetra-packs, pasteurized milk samples and milk shops all were negative for *Mycobacteria*. April 2012 showed the two positive milk samples positive for the *Mycobacteria* while all the thirty three samples were negative. Six tetra-packs, six pasteurized milk packets, nineteen milk samples from milk shops and total four milk samples were observed. One positive sample from milk shops and one from the herd was found positive.

May 2012 results showed three positive samples from the total observed milk samples. Overall three samples were found positive from the milk samples from the herd while all the samples from the different packets were negative for the *Mycobacterium.* From fifteen milk sample three positive samples showed 20% point prevalence. One milk sample from the milk shops and one from the herd was found positive, rests were negative. 9% prevalence from the milk samples and 5.2% milk samples were observed.

Graph showed the incidence of *Mycobacteria* in different months. The higher incidence was on the month of May with overall 3 positive samples. The incidence in this month is higher because of the hot temperature i.e., above 35°C which is ideal temperature for the growth of *Mycobacteria*. Lower incidence was in the month of February because the temperature was not favorable for the growth of bacteria.

Mycobacteria viewed under microscope after acid-fast staining. *Mycobacteria* appeared red due to the acid fast stain while the background is blue due to methylene blue. Colonies of *Mycobacteria* were appeared on Lowenstein Jeensen medium. The colonies have rough and buff in appearance.

Tuberculosis (TB) is a major cause of death and disability in humans, cattle and buffaloes. The causative agent of this disease is a bacterium known as *Mycobacterium*. There are different species of *Mycobacteria* and the most prominent or important are *Mycobacterium tuberculosis* (causing TB in humans), *Mycobacterium bovis* (causing TB in animals) and *Mycobacterium avium* (causing TB in birds). *Mycobacterium* can cross species barrier and can infect human beings through the aerosol, or ingestion of the agent or by the utilization of the products of animals like milk or meat etc. Different types of tuberculosis are meningitis TB, genitourinary TB, gastrointestinal TB, lymphadentitis (lymph) TB, cutaneous TB, uterus ovarian TB, osteo-articular (skeletal-Bone-Joint) TB.

Tuberculosis can be transmitted through coughing, sneezing, by the utilization of contaminated milk or milk products, meat, by handling the infected animal or human, wounds etc [19, 24, 25]. Contaminated milk and milk products can be a source of Tuberculosis. Prevalence of bovine tuberculosis is increasing in Pakistan day by day [26].

In Pakistan, tuberculosis is present in the livestock, wild animals and also in the humans in different forms [27]. Tuberculosis has been reported from time to time in Pakistan. It was reported to be 2.2% in 1969, 7.3% in 1989 and 13.8% in 2001 [28, 29, 30]. Tuberculosis is an important disease both for the human and the animal. Developed

countries adopted the strategies for the removal of this disease but the developing and under developing countries are still suffering from this disease and their incidence rate is also different from region to region [31]. Evangelista,and Anda reported that tuberculosis can be transmitted through the colostrums of the infected cow right after the parturition or through the ingestion of the milk of the infected animal without boiling or proper treatment [19].

The laboratory diagnosis of the tuberculosis is based on the traditional staining method i-e., Ziehl-Neelsen staining or the acid fast staining and then the causative agent i-e., the *Mycobacterium* is cultured on the Lowenstein-Jensen medium. *Mycobacterium tuberculosis* and *Mycobacterium bovis* are clinically and physically same. The difference between them can be found out by the PCR method [32]. The direct smear microscopy is essential as it's the only way to detect the presence of the *Mycobacteria* in the given sample. Direct smear microscopy does not differentiate between the *Mycobacterium tuberculosis* and *Mycobacterium bovis*. They appear in rod-like structures with the blue background due to methylene blue in the microscope. Culture and species difference are often not carried out due to the slow growing behavior of *Mycobacteria*. *Mycobacteria* grows poorly on the Lowenstein Jensen media as it requires 6 to 8 weeks for their proper growth.

During our study overall 4.28% incidence of tuberculosis was observed in milk collected from all resources. Almost same results were observed by different researchers in the same decade. Amin *et al.,* (1992) and Jalil *et al*., (2003) reported 6.9% and 7.3% prevalence of *Mycobacteria* in Lahore respectively. This might be different due to the collection of the samples from different areas. Mumtaz *et al.,* (2008) after only single intra-dermal test reported 9.6% incidence of bovine tuberculosis [33].

Mycobacteria are among those bacteria which are highly resistant to heat and are considered as the most heat resistant pathogens on this basis, but the proper pasteurization i.e., heating the raw milk to 63°C for 30 minutes or to72°C for 15 seconds completely kills the *Mycobacteria* from the milk or completely inactivates it. Therefore to get safe from this dangerous bacteria pasteurized or properly treated milk should be used as the raw milk may be contaminated with *Mycobacterium bovis.*

During the course of study from Jan 2012 to June 2012 overall nine out of two hundred and ten milk samples were found positive. The overall point prevalence was found to be 4.28%. The prevalence of the *Mycobacteria* was more during the hot months that were April, May and June. This may be due to the fact that the temperature in that period of time was nearly or above 37° C which is the optimal temperature for the growth of *Mycobacteria*. The incidence of *Mycobacteria* was low during Jan, Feb and March may be due to the low temperature range during that period of time. The low temperature did not support the production and growth of bacteria. The prevalence of *Mycobacteria* in the milk showed that milk can transmit these causative agents to other animals or other human beings.

Conclusion

The milk samples were examined by culturing and microscopic examination to demonstrate and identify different *Mycobacteria species.* The 4.28% prevalence of *Mycobacteria spp* in milk showed that raw milk is contaminated with many other bacteria and the consumption of the raw milk of the infected animal can transmit bacteria from one animal to another and to humans. So for the better health preventive measures should be used. The most important of which is the consumption of properly treated or pasteurized milk.

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