



## ISOLATION AND IDENTIFICATION OF MICROBIAL CONTAMINATIONS ALONG THE DAIRY VALUE CHAIN IN GWALIOR REGION OF MADHYA PRADESH

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### ABSTRACT

Milk is a natural and rich resource of minerals, vitamins, protein etc. Milk contamination is a very serious public issue, because millions of people especially children all over the world consume dairy products daily. An extensive study was carried out in Gwalior region of Madhya Pradesh, India. The study was to determine the various Milk handling practices and microbial contamination in various milk samples collected from dairy, milk parlor, and small shops. A total of 90 milk samples were collected from four different areas in Gwalior for the isolation and identification of microbes, from February 2016 to April 2016. Previously described standard protocols were adapted for the isolation and cultivation of microbes. Different biochemical assays like IMViC test, Catalase test, Oxidase test, and selective media were used to identify microbes in the milk samples. The results of the study showed that the presence of bacterial contamination (gram positive and negative types of microbes) in raw milk and pasteurized milk result from unhygienic practices during the pre-milking and storage stage. 72 samples were contaminated with *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. However, some more bacterial colonies were also observed, but the results are not stable. Also, drug resistant bacteria against Ampicillin and Tetracycline were reported. On the basis of the present study, it is suggested that dairy farm, dairy shops, and milk parlors must improve the hygienic and storage condition of milk. In addition, public education must be given to all stakeholders in dairy industry on milking and post harvest handling of milk, to curtail the likely losses due to refusal of spoiled milk and milk-borne pathogens resulting from contamination of milk.

**KEYWORDS:** Dairy Milk, Microbial content, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*



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Received on: 29-03-2017

Revised and Accepted on : 15-05-2017

DOI: <http://dx.doi.org/10.22376/ijpbs.2017.8.3.b100-104>

## INTRODUCTION

Unlike in developed countries, the food and dairy industry in India is under developed, and is highly dominated with unpasteurized milk, safety issues, and informal markets. Raw milk is highly known for a major and big issue related to the transmission of milk-borne pathogenic microorganism from animals to humans. Still, most people prefer the raw unpasteurized milk from dairies. Several pathogenic microorganisms are known to cause zoonotic milk borne diseases in humans, such as leptospirosis, tuberculosis, brucellosis Q fever, and campylobacteriosis.<sup>1,2</sup> In recent years, there has been an emergence of some new pathogenic microorganism along the food chain. For example, the emergence of some new milk-borne bacterial pathogens founded with very serious pathogenic microorganism such as *Eschericia coli* O157:H7.<sup>3</sup> The biggest challenge for most of the developing countries still remains, how to identify technological constraints which can improve the dairy value chain. Therefore, some great efforts are needed to distinguish the maximum possible solutions related to the improvement of the milk quality. In this regard, similar studies related to milk need to be carried out in order to provide reliable information which can improve the quality of milk. To this end, molecular based technologies, which are highly sensitive and offers high promising possibility of testing a large number of pathogenic microorganism at various points of the value chain, were developed. The milk dairy and market in Gwalior region is mainly informal and is mainly operated by individual producers. This kind of market has the probability to face a very high possibility and risk of microbial contamination due to the lack of knowledge on microorganism and microbial risks regarding milk handling procedure and consumption.<sup>4,5</sup> Information related to milk handling, techniques, quality assessment and marketing manegment with all the dairy value chain, is insufficient. The informal milk market clearly indicates that the incidences relating to zoonoses, chemical, and drug residues are limited in most areas in India. Gwalior has a wide range of small dairy shops and dairy farmers nearby which were used to produce milk at a subsistence basis and now then producing it for the market basis. Nevertheless, most of the milk related market is still unorganized. Therefore, this study was carried out to explore the possible sources of microbial contamination and the risks factors along the dairy value chains in Gwalior Region. A related study focuses on the presence of pathogenic microorganism in milk, through bacteriological plating. It is anticipated that this study will be used to provide public awareness on the health hazards associated with contaminated milk and some practical measures to solve them.

## MATERIALS AND METHODS

### *Description of the study area*

The current study was conducted in Gwalior region of the Madhya Pradesh. Gwalior region was selected for the study due to its rich and long history of cattle keeping and dairy products, and its market is maintained by non- governmental organizations of small owners and shopkeepers.

### *General survey of dairy farm*

A general survey was conducted along the dairy farm situated in Gwalior region. Several points were identified during the survey including hygienic conditions, storage of milk, water supply, washing of milk cane, vaccination, and training on dairy farming.

### *Sampling and handling of milk samples*

A total of 90 milk samples were collected (including Raw Milk and Packed milk from two reputed brands) from the best possible places in the Gwalior region. About 50 ml of milk sample was collected and kept in sterile tubes, and placed in a cool box with ice packs until it reach to the laboratory. Within 2 to 3 hours, the samples were transported from the field to the laboratory of Amity Institute of biotechnology, situated in Amity University Madhya Pradesh, Gwalior where the samples are stored at 2 to 4 degree temperature till the process is started at the laboratory. The types of milk samples collected are summarized in table 1.

### *Isolation of Bacteria from milk samples*

For the isolation of bacteria, serial dilution method was preferred and it was applied to all of the 90 samples collected from the variou areas of Gwalior Region. In this method, a total of 10 highly sterilized tubes were dispensed with 9 ml of sterilized distilled water. Then, 1 ml of the desiered milk sample was added into the 9 ml strilized distilled water ( $10^{-1}$  dilution). After which 1 ml of that resulting solution was transferred into the second tube containing 9 ml of strilized distilled water ( $10^{-2}$  dilution). This procedure was repeated for the dilution up to  $10^{-8}$ . After completion of the serial dilutions process, 1 ml of the diluted milk sample from the last tube ( $10^{-8}$ ) was spread into a sterile Petri dish. Also, after the incubation period, the number of colony forming unit was counted by using bacterial colony counter. .

### *Identification of microorganism by biochemical test*

For the identification of gram negative bacteria, the culture was confirmed using Gram's staining, cultural, and biochemical examinations. The culture were streaked on MacConkey Agar and incubated at 37°C overnight. The isolated colony was picked for the Gram's stain examination. Further isolates were confirmed by different biochemical test like Indole production, Methyl red, Voges proskaur, Simmon's Citrate Agar, Catalase, oxidase, Nitrate reduction, Urease production. The identities of the isolates were also confirmed based on positive results for the growth pattern on MacConkey Agar and beta haemolytic patterns on blood agar, and slide agglutination test.

### *Multi Drug Resistance testing on various milk samples*

Agar disk diffusion method was used to examine multi drug resistance bacteria.<sup>6</sup> After an overnight incubation on muller hinton agar plates, isolates were examined and the diameters of the zones of inhibition were measured using millimeter scale. The zone diameter were categorized (susceptible or resistant) with the help of the interpretation table of Becton Dickinson Microbiology Company, USA.

## RESULT AND DISCUSSION

### Isolation and Identification of Bacteria from collected milk sample

A total of 90 milk samples (including pasteurized and raw milk) were collected from different milk parlour, dairy, and small shops (Table 1). After culture of these samples, 72 bacterial isolates were observed and sub-cultured for further analysis. However, only 03 bacterial

isolates were identified on the basis of different biochemical analysis. The remaining samples of bacterial isolated were not identified because the results are not reproducible. One the basis of biochemical analysis including IMViC test, Oxidase test, Catalase test etc., which were used to demonstrate the presence of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* in the different milk samples.

**Table 1**  
**Details of contaminated milk samples obtained from different places in Gwalior region**

S.no	Area	Collection centers	No. of Samples	No. of contaminated milk samples
1	Thatipur Zone	Dairy	10	10
		Milk parlor	06	01
		Small shops	08	08
2	Morar Zone	Dairy	11	11
		Milk parlor	05	02
		Small shops	10	10
3	Phool bagh area	Dairy	04	04
		Milk parlor	06	02
		Small shops	08	08
4	Maharaj Bada area	Dairy	04	04
		Milk parlor	08	02
		Small shops	10	10
Total			90	72

### Distribution of observed Bacteria

Milk samples collected from different places like milk parlour, home suppliers, and small dairy owners were used in this study (Table 1). Out of 90 samples collected from different centres, 29 bacterial contaminates were observed from small dairy (100%), 36 from shops (100 %), and from 7 milk parlour (28%).

### Antibiotic susceptibility

Antibiotic resistance has increased worldwide due to excessive use of antibiotics without sufficient knowledge, which is leading to failures in the treatment of human infectious diseases. The Kirby-Bauer disk

diffusion test was a very simple and effective method, to determine if the isolated organisms were susceptible or resistant. All isolated bacterial samples were further subjected to antibiotic susceptibility tests. Four antibiotics, namely Ampicillin, Tetracyclin, Ciprofloxacin and Chloramphenicol, were used to detect drug resistant bacteria. Large numbers of bacterial isolates, identified from small dairy and shops were resistant to Ampicillin and Tetracyclin. However these isolates were sensitive to Ciprofloxacin and Chloramphenicol. Small proportion of bacterial isolates identified from pasteurized milk were also resistant to ampicillin (Table 2).

**Table 2**  
**Antibiotic susceptibility test with identified Bacteria**

S. No.	Milk samples	Name of isolated bacteria	No. of isolated Bacteria from milk samples	Antibiotic resistance			
				AMP	TET	CIPRO	CHLORO
1.	Raw Milk	<i>Staphylococcus aureus</i>	55	+	+	-	-
		<i>Escherichia coli</i>	40	+	+	-	-
		<i>Pseudomonas aeruginosa</i>	28	-	-	-	-
2	Pasteurized milk	<i>Staphylococcus aureus</i>	7	+	+	-	-
		<i>Escherichia coli</i>	7	+	+	-	-
		<i>Pseudomonas aeruginosa</i>	-	-	-	-	

+ Growth of the bacteria  
- No growth of the bacteria.

## DISCUSSION

This study focused on evaluating the bacterial contamination in pasteurized milk supplied by different cooperative society and small dairy, in Gwalior region in Madhya Pradesh, INDIA. There are possible risk factors available for the microbial contaminations along with the dairy value chain which were explored; also the involvement of some of the pethogenic microorganisms as main milk-borne pathogens was explained using various biochemical method and other lab techniques.

This was as a result of the fact that, milk produced in Gwalior region by the informal sector is not controlled by any agency and such milk may cause a big health problem to the human society due to contamination with pathogens. In general, it is established that, animal feeding and housing, animal health and management, related practices of milk storage, harvesting, transportation, and retailing, predispose the milk to microbial contamination. The worldwide cleanliness at milking is distinguished to have an effect on the most numbers of microorganisms in various milk and its

products. It is highly recommended that before milking, it is very important that the animal house should be pretty much cleaned; the udder should be properly washed and dried before the process of milking. After milking, dipping of teats in suitable disinfectant is necessary to fully control the entry of microorganisms which comes through the teat canal. The basic workforce and the required tools must be clean. During this study, it was reported that, 70% of dairy owners did not clean their hands with suitable sanitizers or soap solution. Also, they did not wash cow teats and did not properly clean the animal houses before milking. Certainly, the habit of hand milking with unwashed hands practiced by farmers, optimistically point out that the microorganisms on hands surface could be source of contamination to the milk. One of the previous study by in Tanga reported similar observations.<sup>7</sup> Furthermore, some other studies in Zimbabwe, and Ghana reported same unhygienic practices along with the milk value chain.<sup>8,9</sup> It was also reported that the basic containers which was used during the process of storage, milking, and distribution were thin necked plastic containers, which are sometimes very complicated to wash. Thin necked plastic containers are not that much easy to wash particularly in the inner corners and this problem lead to a very high possibility of sticking the milk residues. In such a situation, microorganisms may contaminate the milk. Previously it was reported that the problem of plastic containers which increased microbial counts in milk is very frequent.<sup>10,11</sup> Moreover, it was stated that from the number of farmers in the Gwalior Region that were interviewed, 50% did not get any training related to livestock handling and many other milking hygienic practices. Furthermore; many of the farmers did not consult with a veterinary doctor when their animals were unwell; while some did not know the significance of using various feed supplements and few of the others did not know the value of regular check of there animal health. The presence of high bacterial load in the milk may not be very much surprising since the untreated raw milk will be harvested from various dirty animals of dirty animal houses, the unhygienic surroundings and basic milk handling may have became the main issue of contamination in raw milk.<sup>12</sup> The results related to the present study are in similar to previous work, done in Tanzania, in that work most of the tested samples had a higher bacterial count above the standards.<sup>13,14</sup> Some of these findings were also compared with studies done in Ghana, Ethiopia which was different from another study done in Sudan.<sup>14-16</sup> In addition, the identified bacteria in the milk samples were *S. aureus* and *E.coli*, consequently indicates a safe risk for human life. From the pasteurized packed milk samples collected from various milk parlor and local shops, gram-positive bacteria *S. aureus* were identified. This may be because of improper pasteurization or effectiveness of pasteurization or unhygienic conditions at the processing plant. It was also reported that if the load of microorganisms was very high in raw milk samples, then

bacteria could survive during pasteurization process.<sup>17</sup> Therefore, keeping packed milk at room temperature for several hours in milk parlor or shops may lead to spoilage of milk. Some bacteria, specially thermostable bacteria, can survive at high temperature because many of them can produce extracellular thermostable proteolytic and lipolytic enzymes; thus affecting the shelf life and quality of milk and milk products during storage<sup>18</sup>. Furthermore, these isolates including *S. aureus*, *P. aeruginosa* and *E.coli*, samples were tested for multidrug sensitivity. The *S. aureus* and *E.coli* strain was reported to be resistant to ampicillin and Tetracycline antibiotics. It was found in previous studies that antibiotics are widely used in animal food and treatment for the prevention of bacterial infections without any consultation of expert. It is a very serious issues related to human health and food safety. These results are similar to previous studies.<sup>19,20</sup>

## CONCLUSION

Milk is an excellent nutrient medium for the growth of pathogenic bacteria, which can contaminate milk from various sources at different critical control points. The result of bacteriological assessment of raw milk contaminants at different critical control points in this study showed that, diverse environmental bacteria could contaminate milk. A high numbers of *S. aureus* and non *S. aureus* isolates may be due to the use of contaminated water, absence of detergents and/or disinfectants to wash udders and milk equipment and environmental contamination from dusty road during transportation. It is recommended that (1) educating the dairy farm owners about microbial safety procedures from the point of udder sanitary processes to the sanitary practices at collection centres (2) Short term Training of dairy owners regarding basics of dairy industry, new equipments, their uses (3) Set up of cooling systems at milk collection sites (4) good transportation systems will also play key roles in reducing contamination of the milk.

## ACKNOWLEDGEMENT

We wish to express our sincere acknowledgement to Dr. Ashok Kumar Chauhan, President, RBEF parent organization of Amity University Madhya Pradesh (AUMP), Dr. Aseem Chauhan, Additional President, RBEF and chairman of Amity University Gwalior Campus, Lt. Gen. V.K. Sharma, AVSM (Retd.), Vice Chancellor of AUMP Gwalior Campus, for providing necessary facilities, their valuable support and encouragement throughout the work.

## CONFLICT OF INTEREST

Conflict of interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript