DOI:https://doi.org/10.54393/pbmj.v5i5.469

PAKISTAN BIOMEDICAL JOURNAL

https://www.pakistanbmj.com/journal/index.php/pbmj/index

Volume 5, Issue 5 (May 2022)

Original Article

Assessment of Microbiological Quality of Raw Milk and Identification of Pathogenic Bacteria

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ARTICLE INFO

Key Words:

Microbial Quality, Milk, Mastitis, Animal Diseases, Contamination

How to Cite:

Afreen, A., Ashraf, A. ., & Chaudhry, A. (2022). Assessment of Microbiological Quality of Raw Milk and Identification of Pathogenic Bacteria: Microbiological Quality of Raw Milk. Pakistan BioMedical Journal, 5(5). https://doi.org/10.54393/pbmj.v5i5.469

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Received Date: 21st May, 2022
Acceptance Date: 27th May, 2022
Published Date: 31st May, 2022

ABSTRACT

Milk contains important nutrients such as minerals, vitamins, proteins and lipids and are consumed by all age group of humans around the world. It is impossible to avoid contamination of milk with micro-organisms because presence of nutrients therefore quality of milk can be determined by the microbial content in milk. **Objective:** To investigate the microbiological quality of raw milk. **Methods:** In the present study, there were 30 cow milk samples collected from different dairy farms of Lahore. Firstly, a surf field mastitis test was performed for detection of clinical and sub-clinical mastitis. The microbial isolation was performed by microbial culturing and biochemical tests and antibiotic sensitivity test was performed for isolated bacteria. These isolated bacterial DNA was extracted and amplified by *16S rRNA* PCR. The precipitated amplicon was sequenced by *16S rRNA* sequencing. The results were evaluated statistically to check the level of significance among them. **Results:** The *Chi-square* values of catalase test, oxidase test, indole test, methyl red test, Voges Proskauer test and triple sugar iron were 12.42, 13.77, 8.77, 9.02, 10.67 and 4.29 respectively and the *p-values* were 0.034, 0.031, 0.042, 0.039, 0.044 and 0.056 respectively on MacConkey Agar. The *Chi-square* values of catalase test, oxidase test, indole test, methyl red test, Voges Proskauer test and triple sugar iron were 12.44, 11.98, 9.38, 7.02, 14.22 and 10.43 respectively and p-values were 0.034, 0.045, 0.039, 0.012, 0.022 and 0.053 receptively on Mannitol salt Agar. The *Chi-square* and *p-values* of gram staining bacteria were 13.99 and 0.034 respectively and showed the significant relationship among them. Mastitis test were presented the value of *Chi-square* 17.86 and *p-value* 0.029. The *ANOVA* table on DNA isolation method were exposed the highly significant relationship among the variables. **Conclusions:** There was a significant association between different treatments. Different pathogens can grow in milk and milk products and produce toxic metabolites. Products that are contaminated by these toxic metabolites when consumed may results in food poisoning.

INTRODUCTION

Milk contains important nutrients such as minerals, vitamins, proteins and lipids and are consumed by all age group of humans around the globe [1, 2]. It is impossible to avoid contamination of milk with micro-organisms because presence of nutrients therefore quality of milk can be determined by the microbial content in milk [3]. Raw milk when leaving the udder has very low microbial contamination but due to possible exposure to various environmental contaminants microbial load immediately increases after milking [4]. The microbial contamination can be occurred from ill cow's udder and teat, unhygienic milking utensils, poor milking practice, and low maintained transportation [5,6]. The regular request of bacteriological analysis of the quarter milk samples is hindered by financial considerations. Alternative parameters are also used to define patterns in the health of udder production in a dairy herd, as these limits suggest inflammation [7]. The manufacturer of such products, usually follow traditional procedures and shows lack of concern about quality of milk used. These practices cause harmful microorganisms to gain access in milk-based products [8,9]. Effective and good hygienic practice at farm can minimize the microbial contamination in milk before transporting to the markets, screening of milk is important for protection against milk

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borne infections [10]. The temperature at which milk is kept after milking disturbs the quality of milk and likewise influence the production of microbial growth [11]. To prevent milk contaminations, it is necessary to boil or pasteurize the milk after milking or cool immediately and keep in a clean environment. The pathogens in milk gain entry mainly to low animal hygiene and poor milking [12, 13]. Therefore, the main purpose of this research was to determine bacterial load in raw milk samples from different farms and to isolate different type of bacteria and its strains and to find out antibiotic resistance bacteria in mastitis positive and negative samples.

METHODS

A total of 30 cow milk samples from dairy farms of Lahore were collected. Firstly, cow teats were sterilized and milk was collected from each teat of a cow in a sterilized container. All the containers were stored at 4°C for further processing. The milk sample collection procedure was performed according to Quinn *et al.* [14].

Mastitis screening test: The surf field mastitis test was performed for finding of subclinical and clinical mastitis. Milk sample was mixed with surf detergent in the Petri plate and stirred with a sterilized glass rod in a gentle circular rotation. The appearance of clots and gel-like structures was observed [15].

Microbial Culturing: Microbial culturing was performed directly on nutrient agar and after the growth of samples on nutrient agar, morphological characters of the colonies were observed by direct examination of colonies. MacConkey agar and mannitol salt agar were used for differentiation in growth according to gram-positive and gram-negative bacteria, Gram staining was done on sterile glass slide for all isolated colonies [16].

Biochemical tests: For the biochemical identification of bacteria catalase, oxidase, indole test, methyl red test, Voges Proskauer test and triple sugar iron tests were performed for characterization of bacteria [17].

Antibiotic Sensitivity test: The antibiotic sensitivity tests of the bacterial isolates were performed according to the NCCLS (National Committee for Clinical Laboratory Standards) method by using Kirby Bauer disk diffusion test on Muller Hinton agar. Mueller Hinton Agar was autoclaved, allowed to cool down and poured into the sterile petri dishes and the plates were inoculated by using a sterile swab, a total of 48 plates were swabbed for 48 isolated colonies and antibiotics discs (Tazobactam, Amikacin, Gentamicin, Tobramycin, Imipenem, Clavulanic acid, Ceftriaxone, Levofloxacin, Doxycycline Linezolid, Ampicillin, Vancomycin, Nitrofurantoin, Fosfomycin, Meropenem, Trimethoprim, Polymyxin B, Nalidixic acid) were placed on the plates at a distant position. All plates were incubated at 37°C for 24hr. The zone of inhibition was measured and results for each isolate was concluded as susceptible, intermediate, and resistant based on the standards of inhibition zone given by CLSI (Clinical and Laboratory Standard Institute).

Molecular Identification: Extraction of Bacterial DNA was performed by using two methods, the QIAGEN Kit method for gram-positive bacteria and PEG (polyethylene glycol) buffer method for gram negative bacteria. QIAGEN kit method is column-based DNA extraction followed by manufacturer's protocol. For gram-negative bacterial DNA isolation boiling method was used, in this method, PEG polyethylene buffer was used. PCR was performed by using 16SrRNA primers, Forward primer: 27F 5'-AGA GTT TGA TTC TGG CTC AG-3' and Reverse primer: 515R 5'-TTA CCG CGG CTG CTG GCA C-3'. The PCR amplicons were placed at 4°C and then characterized by agarose gel electrophoresis.

Sequencing and Sequence Analysis: The amplified 16S rRNA fragment was precipitated and sequenced using DNA sequencing services of First Base Company Lahore. 16S rRNA sequences were analyzed by using BLAST (Basic Local Alignment Search Tool) available from the website of NCBI (National Center for Biotechnology Information) to identify the similar matches with existing reference sequences.

Statistical Analysis: *Chi- Square* value and *P value* were calculated by applying *Chi-Square test* on results. *ANOVA test* was applied on antibiotics sensitivity test results. All statistical values were interpreted and significant and non-significant associations were recorded.

RESULTS

Mastitis test: When mastitis test was performed in total 30 raw milk samples 14 samples were mastitis positive and 16 samples were mastitis negative, in 16 negative samples, 8 samples showed growth on nutrient agar and 8 sample did not show any growth on nutrient agar.

Microbial Culturing: Out of 30 samples 22 showed growth on nutrient agar and 8 did not showed any growth. Total 48 colonies were selected on nutrient agar.

Biochemical Characterization of the Bacterial Isolates: In gram staining results in total 48 isolated bacteria 25 were gram negative and 23 were gram-positive.

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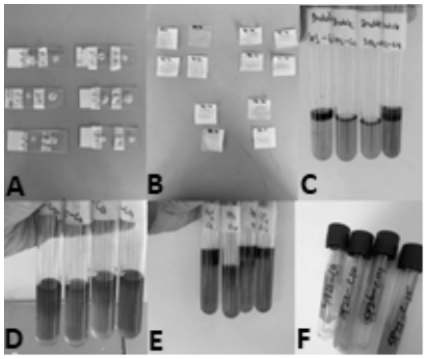

Table 1: Numeral of splenic artery with its primary segmental branches

Antibiotics Sensitivity test: Antibiotics sensitivity test was performed. In results, 23 isolates were sensitive to tazobactam, 14 isolates were intermediate to Fosfomycin and 19 were resistant to gentamicin.

16s rRNA sequencing results: The sequencing results were analyzed by BLAST on NCBI. On BLAST 16 bacteria were matched with *E. coli* with identities of 99%, 8 were matched with *Klebsiella spp.* with identities of 99 %, 1 was matched with *Pseudomonas spp.* with identities of 99%, 17 were matched with *S. aureus* with identities of 99%, 6 were *Streptococcus spp.* With identities of 89% (Table 1).

Microbial Culturing base results	Number of isolated bacteria	16s rRNA Sequencing Results	Query cover	Percentage identical	Accession No by NCBI
Escherichia coli	16	Escherichia coli strain	100.00%	99.60%	MT230530.1
Klebsiella	8	Klebsiella pneumoniae strain sctcc18	100.00%	99.84%	HQ622339.1
Pseudomonas	1	Pseudomonas sp. KLEPS3	100.00%	99.81%	JQ910871.1
Staphylococcus aureus	17	Staphylococcus aureus subsp. Aureus	100.00%	99.45%	CP054876.1
Streptococcus	6	Streptococcus sp	100.00%	88%	EU826665.1

Table 1: Sequencing results matched on BLAST

In the mastitis test result chi-square test was applied to check significant association (table 2). The chi-square value was recorded as 17.86 and the p-value as 0.029 which shows there is significant association between them as the p value was less than 0.05.

Mastitis Test	Growth of samples on Nutrient Agar		Total
	Showed no growth	Showed growth	
Mastitis Negative	8	8	16
Mastitis Positive	0	14	14
Total	8	22	30

Table 2: Mastitis Test
Chi-square value = 17.86, P-value = 0.029

Biochemical tests Association with Isolated colonies on Differential Media: The p-value was calculated between the association of biochemical tests and colony characteristics on MacConkey agar and mannitol salt agar (Table 3). In case of biochemical test (catalase, oxidase, indole, MR, VP) association with isolated colonies on MacConkey agar and mannitol salt agar p-value was lesser than 0.05 which showed the important association it means the result of all biochemical test of isolated bacteria had a significant difference, each colony showed a different result, and it showed the different types of gram-negative and gram-positive bacteria. In the case of the triple sugar iron test association with isolated bacteria is non-significant because the p-value was more than 0.05 it shows that all bacteria isolated on MacConkey agar had the same result. Only gram-negative bacteria can grow on MacConkey agar and all these gram-negative bacteria were triple sugar iron test positive and bacteria that can grow on mannitol salt agar are gram-positive and these are always negative results for triple sugar iron test.

Biochemical tests	Colony characteristics on MacConkey agar		Colony characteristics on mannitol salt agar	
	Chi-square value	P-value	Chi-square value	P-value
Catalase Test	12.42	0.034	12.44	0.034
Oxidase Test	13.77	0.031	11.98	0.045
Indole Test	8.77	0.042	9.38	0.039
Methyl-Red Test	9.02	0.039	7.02	0.012
Voges-Proskauer Test	10.67	0.044	14.22	0.022
Triple Sugar Iron	4.29	0.056	10.43	0.053

Table 3: Biochemical Tests Result

ANOVA test was applied on results of antibiotic sensitivity test. We compared our result with the p-value if the p-value >0.05 it showed significant result. If the p-value is less than 0.01 then it means the results are highly significant from the table 6 the p-value for the replication is 0.004 and for the treatment is 0.030 that are less than 0.05 it means results are significant (Table 4).

Source of Variation	SS	DF	MS	F	P-value
Antibiotic Discs	51.59	7.00	7.37	15.00**	0.00
Samples	32.27	24.00	1.34	2.74**	0.00
Error	82.53	168.00	0.49		
Total	166.39	199.00			

Table 4: ANOVA Table
(SS: sums of square, df: degree of freedom, MS: mean square value, F: variation between sample means), *= Significant **= Highly significant

DISCUSSION

The aim of this study was assessment of microbial quality of raw milk and detection of different strains of bacteria. At the time of milking, milk has a low bacterial count but after milking the bacterial load increases, due to various external and internal contaminants [6]. Maintaining good quality of milk is a main challenge in dairy sectors worldwide, where production of milk and its products take place in unhygienic conditions [18,19]. In the present study 30 cow milk samples were collected from dairy farms in Lahore and raw

DOI:https://doi.org/10.54393/pbmj.v5i5.469

milk quality was checked by culturing milk samples and different types of bacteria were isolated. Out of 30 cow milk samples 8 samples did not show any growth and 22 showed growth, 48 bacterial colonies were selected from 22 samples including 25 gram-negative bacteria and 23 gram-positive bacteria. However, in the study of bacteriological quality of raw milk, there were 25 different areas in Guwahati city selected and total 200 raw milk samples collected to check microbiological quality of raw milk. The total coliform count (TCC) and total viable count (TVC) was estimated and results showed highly significant differences in total coliforms count and viable count in different areas around the city [20]. In current study, 14 samples were mastitis positive and 16 samples were mastitis negative. To check the association among the growth on nutrient agar chi-square test was applied in mastitis positive and negative samples 48 bacteria were isolated including E. coli, Klebsiella spp., S. aureus, Pseudomonas spp., and Streptococcus spp. In another study species composition of microbiota of cow's udder and raw milk quality was detected. The milk from animals with clinical and subclinical mastitis had higher number of somatic cell count and in mastitis positive samples 16 species of bacteria were isolated. In microbial culture, the microflora included S. aureus, S. hyicus spp., (Staphylococcus hyicus), S. agalactiae (Streptococcus agalactiae), S. lentus (Staphylococcus lentus), and S. intermedius (Streptococcus intermedius) [21]. In the results most of isolated bacteria showed resistance to gentamicin. Comparative studies on resistance profiles of E. coli isolated from goats' milk are very rare and found approximately 18.2% of the isolates to be resistant against ampicillin [22]. According to procedure involving Triton X-100-based pretreatment and an inhibitor removal resin was superior to all other methods tested in terms of DNA yield, sensitivity, ease of sample handling, time efficiency, and cost per sample. But in present study two methods were used, Qiagen kit method and PEG buffer method was used for bacterial extraction. Gram positive bacteria cell wall have a thicker layer of peptidoglycan and their DNA cannot extracted by Boiling method by using PEG buffer, therefore special kits such as Qiagen kit used for gram positive bacteria extraction. A research of "Microbiological safety concerns of raw milk" showed that microbial contamination of milk was raised from unhygienic conditions coupled with improper processing and handling result it unsafe products causing several diseases outbreaks [23]. Microbiological analysis of raw milk indicated presence of pathogenic organisms like coliforms [18] S. aureus [21], E. coli [24], E. aerogenes spp., (Enterobacter aerogenes)., Salmonella typhi [25] Salmonella spp. from India, Klebsiella spp., Proteus spp., Enterobacter spp., Mycobacterium spp. from Ghana [25] E. coli, Aeromonas, Salmonella from Bangladesh. E. coli, Bacillus spp., Clostridium spp., coliforms from Pakistan, also different species of different kinds of bacterial are also seen in the milk in different countries. The most common microorganisms associated with the environment are E. coli and S. uberis (Streptococcus uberis). The vast majority of mastitis of bacterial origin (80% of cases) is caused by five species of bacteria, namely E. coli, S. uberis, S. aureus, S. (Streptococcus dysgalactiaes) and S. agalactiae [24]. The study indicated that the dominant microbial flora associated with raw milk samples in and around were in the order of Lactobacillus spp. > S. aureus > E. coli > Bacillus spp. > Pseudomonas fluorescens > Salmonella spp. > among the isolated pathogens. The presence of those bacteria in milk suggested contamination from various sources, such as animal, human, environment, utensils and others [24]. In current study total 48 bacteria were isolated from mastitis positive and negatives samples, all these bacteria were identified by microbial culturing and biochemical tests and confirmed by 16S rRNA sequencing, including 16 E. coli, 8 Klebsiella spp., 1 Pseudomonas aeruginosa, 17 S. aureus, and 6 Streptococcus spp.,

CONCLUSION

Different pathogens can grow in milk and milk products and produce toxic metabolites. Products that are contaminated by these toxic metabolites when consumed may results in food poisoning. It is main challenge to monitor the microbiological quality of milk to ensure its safety for consumers. In present study on the basis of culturing and biochemical identification, 16 E. coli, 8 Klebsiella spp., 1 Pseudomonas spp., 17 S. aureus, and 6 Streptococcus spp., were detected in mastitis positive and negative milk samples and it was further confirmed by 16S rRNA sequencing. Different bacteria isolates found in both mastitis positive and mastitis negative samples. Bacterial isolates antibiotic resistance profile was checked by antibiotic sensitivity test. In mastitis negative samples bacteria also isolated it means contamination can occur by environment e.g., milk handler, water that used for washing milk contact surfaces. Hands of the milk handler are the main reason affecting microbial quality of raw cow milk. Hence, measures should be taken to improve the attitude and educational status of milk handlers and the quality of water to enhance.

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