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Detection of Pathogenic Bacteria *Staphylococcus aureus* and *Salmonella* sp. from Raw Milk Samples of Different Cities of Pakistan

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ABSTRACT

Food-borne diseases are the main public health problem throughout the world. Milk is important component of human diet including fats, proteins, vitamins and minerals. It is a best source of calcium and phosphorus. Different types of pathogenic bacteria like *S. aureus* and *Salmonella* enter in milk and then multiply, after multiplication they become active in causing diseases. These bacteria create serious problems for human health. This study aimed to isolate and identify pathogenic bacteria *Staphylococcus aureus* and *Salmonella* from raw milk samples of different cities of Pakistan. Primary screening of raw milk samples was done on the basis of morphological, cultural and biochemical techniques. The final identification was made using 16SrRNA sequence analysis. A total of 200 raw milk samples were collected from different cities of Pakistan. Selective medium xylose lysine deoxycholate agar (XLD) and Mannitol salt agar were used for the identification of *Salmonella* sp. and *S. aureus*. *Staphylococcus aureus* produced yellow colonies with yellow zones on Mannitol salt agar. *Staphylococcus aureus* exhibited gram-positive character with purple coloration and it was detected as cocci-shaped. Biochemically 91 (45%) samples enhibited Catalase, Coagulase, DNase, Urease, Citrate, fermentation tests positive and indole, oxidase and H_2S tests negative with nonmotile character, indicating the presence of *Staphylococcus aureus*. *Salmonella* sp. was detected as gram negative rods with pink coloration on gram staining. Biochemically 87 (43%) samples revealed catalase, citrate, H_2S and fermentation tests positive while oxidase, DNase, Indole and urease tests negative, indicating the presence of *Salmonella* sp. in

these samples. Of the 200 samples tested, 43% were positive for S*almonella*, while 45% samples were contaminated with *S. aureus*. The 16SrRNA sequence analysis confirmed the results of biochemical and cultural characterization by depicting 99% identity of samples with *S. aureus* and 98% identity with *Salmonella* spp. The occurrence of high percentage of these pathogenic bacteria in raw milk may be linked to its contamination at the time of collection, processing, strorage and distribution. This quantitative data could be utilized to better establish the appropriate levels of protection for raw milk, dairy products and processing technologies.

1. INTRODUCTION

Milk is complex ecosystem for various microorganisms as well it is essential component of diet or best medium for bacterial growth. Traditional consumption of raw milk poses a serious threat to health of people [1]. Bacterial pathogens such as *Mycobacterium tuberculosis*, *S. aureus*, *Salmonella* spp., *Coxiella burntinii*, *Campylobacter* etc. are major food borne pathogens. Although pasteurized milk is also available in markets, yet raw milk and its products are consumed by a large population of villages. Investigation of pathogens in raw milk and its products is an important step in quality assurance of milk and milk related products [2, 3]. Several diseases are caused due to consumption of raw milk and its products. Brucellosis is a disease transmitted to humans by animals due to the consumption of raw milk and its products [4-6]. Bacterial gastro-intestinal infection is caused by *Campylobacter jejuni*. It is caused by contaminated water. Bacterial gastro-intestinal infection may also caused by addition of contaminated water in milk. Sometimes diseased animals like cows and buffaloes also secrete some pathogenic microorganisms in their milk. *S. aureus* is mostly present in unpasteurized milk and such contaminated milk products cause food poisoning and gastrointestinal illness, which are responsible for broad variation in texture and taste of milk and produce enzymes which can facilitate bacterial attack and proliferation in the body of host. Several food borne outbreaks have been associated with the consumption of unpasteurized milk [7].

Staphylococcal food poisoning occurs by the consumption of toxins produced by *S. aureus*. Food handlers carry enterotoxin-producing *S. aureus* in their noses or on their hands and other main source of food contamination via direct contact or through respiratory secretions [8]. In Pakistan, approximately 2 billion litre milk is produced annually. Further 75% of the milk is collected from cows in rural areas and 25% from other animals. Milk is consumed in almost 30% of the market products such as chocolate, ice cream, butter and cheese etc. In many countries, 5% - 10% milk provides total calories of the daily human diet. Consumption of contaminated milk in different areas of Pakistan results in diseases such as typhoid fever and several other types of infections. The major causative agents of these diseases are different pathogenic micoorganisms e.g. *Salmonella* and *S. aureus* etc. Central disease control (CDC) reported cases (1998-2011) showed that milk borne pathogenic bacteria caused different types of diseases. About 148 out breaks occurred due to the consumption of raw milk, 2384 illnesses, 284 Hospitalization and 2 deaths occurred. About 1.4 million of the annual food borne illnesses in the US is caused by *Salmonella* sp. (2000-2005), which were reported due to infected raw milk [9].

Food borne infection has been increasing day by day due to different types of food contaminating bacteria [10]. These diseases are among the main public health problems throughout world. *Salmonella* is a worldwide problem in public health sector. 3 - 4 million cases have been reported annually due to this pathogen. *Salmonella* is a gram negative and rod shaped bacteria with incubation period of 12 - 36 hours. *Salmonella* species cause gastroenteritis infection. The presence of *Salmonella* and other human pathogens in unpasteurized milk causes public Health Hazards. *Salmonella* food poisoning is the most common bacterial and widely distributed disease worldwide, estimated to cause 3 million deaths [11]. Pathogenic *Salmonellae* ingested in food survive passage through the gastric acid barrier and invade the mucosa of the small and large intestine and produce toxins. Invasion of epithelial cells stimulate the release of pro-inflam-

matory cytokines which induce an inflammatory reaction. The acute inflammatory response causes diarrhoea and may lead to ulceration and destruction of the mucosa. The bacteria can disseminate from the intestines to cause systemic disease.

Different types of pathogenic bacteria were detected in a study of Malyer city in Iran. They had indicated the presence of *E. coli* (75%), *S. aureus* (52%), *Klebsiellia* (36%), *proteus* (4%) in raw milk. They revealed that sources of contamination of milk may include animal, human, environment, and utensils etc [12]. Donkor *et al.*, (2007) also demonstrated the presence of different pathogenic bacteria in raw milk of two cities of Ghana. They revealed the percentages of *E. coli* (21.1%), *klebsiella* spp. (16.7%), *Bacillus spp* (11.5%) in unpasteurized milk [13]. Daka *et al.*, (2012), examined cow milk sample to detect antibiotic resistance against the *Staphylococcus aureus* [14]. They collected 160 milk samples and used different biochemical tests for the identification of pathogenic microorganisms. Most of samples were contaminated with *S. aureus* and all strains were resistant to drugs like penicillin, Amoxicillin, Ciprofloxacin, and erythromycin. If food is stored at room temperature, the micro-organisms multiply in food and produce different types of toxins. *S. aureus* produces six different types of enterotoxins (A, B, C, C2, D and E). Mostly food poisoning occurs due to enterotoxin A. These enterotoxins are heat stable. *Staphylococcal* enterotoxins acts as super antigens, binding to MHC II molecules and stimulating T cells to divide and produce lymphokines such as IL-2 and TNF-alpha, which induces diarrhoea. The toxin acts on the receptors in the gut and sensory stimulus is carried to the vomiting center in the brain by vagus and sympathetic nerves. Incubation period of *S. aureus* is 1 - 6 hours. *S aureus* is a most important food born pathogenic organism causing usually skin infections as well as other diseases like boils, cellulite, toxic shock syndrome [15].

Improvement of hygienic conditions during handling, distribution and storage of raw milk, can control these pathogenic bacteria to a larger extent [16]. Ali *et al.*, (2011) estimated different pathogenic bacteria in cow milk in Khartoum State. 100 samples were collected from different places of Khartoum and Omdurman. Pathogenic bacteria were identified by culturing on media and further confirmation of bacteria was made by biochemical tests. 63% milk samples exhibited the presence of *E. coli*. The high numbers of milk samples were contaminated with *E. coli* from Khartoum. The highest number of coliform was found in milk from Khartoum farms with 15.0×10^4 cfu/mL, while the lowest number count of 3.857 ± 0.02 log10 cfu/mL was detected in milk obtained from the Omdurman farms [17]. Srujana *et al.*, (2011) performed a study for the analysis of different pathogenic microbes in raw and pasteurized milk [18]. They indicated the pathogenic contamination (*Lactobacilli*, *Staphylococcus aureus*, *E. coli*, *Bacillus subtilis*, *Salmonellatyphi*, and *fecal coli forms*) of most of the raw milk samples. Among the raw milk samples only 19% samples were best for human consumption and 28% were of very poor quality. Among the pasteurized milk samples, 81.9% of samples were good for human consumption. Bassam *et al.*, (2014) had also demonstrated the presence of *S. aureus* in raw milk in a study in Barash [19]. About 22 samples were found to contain *S. aureus* and it was found sensitive to different antibiotics eg. kanamycin, azithromycin, vancomycin, streptomycin and resistant to oxcillin and ampicillin.

Present study was designed to isolate pathogenic bacteria (*Salmonella* spp. and *S. aureus*) from unpasteurized milk of different cities of Pakistan. This research may be helpful for policy makers to adopt safety measures and set some rules and regulations for the prevention of food borne diseases. This may help to control the outbreak of harmful diseases due to raw milk and it's products consumption.

2. MATERIALS AND METHODOLOGY

2.1. Collection of Milk Samples and Preparation

Two hundred raw milk samples were collected from different cities of 3 provinces (Punjab, KPK and Sindh) as well as Kashmir in Pakistan. Samples were collected from February 2015 to July 2015, in sterilized falcon tubes and were directly sent to the laboratory under cold condition. Then these were analyzed within 24 hours. Experiments were conducted in research labs of University of Haripur. Sample preparation was done through serial dilutions in a laminar air flow. Five sterile test tubes were taken for each sample, marked and labelled for each sample. About 1 ml of milk sample was taken in 9 ml of peptone wa-

ter in a first tube and is votexed. This stock solution was then diluted serially upto the dilution of 10^{-5} and 0.1 ml of diluted sample was inoculated on the sterile nutrient agar plates then incubated at 37˚C for 24 hours in an incubator. For the isolation of pure cultures of bacterial strains, the sub culturing was done by using the sterile loop to pick the bacterial colonies having clear boundary and were streaked on the fresh nutrient agar plates by using streak plate method and were incubated at 37˚C for 24 hours.

2.2. Isolation & Identification of *S. aureus* and *Salmonella* sp. by Cultural, Morphological and Biochemical Methods

The selective medium used of isolation of *S. aureus* was Mannitol salt agar. A loopful of inoculum from nutrient plate was streak on Mannitol salt agar and incubated for 48 hours at 37˚C. After isolation of bacterial strains on Mannitol salt agar, strains were identified and characterized by morphological and biochemical tests using Begay's manual as a reference [20]. Microscopic and biochemical tests were performed to recognize the isolated strains. For microscopic identification gram staining technique was performed. Isolated bacterial colonies were observed and analyzed for morphological and biochemical characteristics of *S. aureus*. The smear was prepared from isolated culture on clean glass slide and stained with gram staining. The stained smear was observed under microscope at oil immersion 100× lens. First added one drop of water on glass slide. A loopful of bacterial colony was added from pure culture of *S. aureus* and mixed gently. The smear was made thin then air dried or fixed with heated flame. Added crystal violet on the slide for 1 minute then washed with tap water. After washing, added gram iodine for 1 minute then washed with tap water. Added decolorizing agent on slide waited for 15 second then washed with tap water. Lastly added safranin for 15 seconds then washed with tap water. After gram staining slide was air dried then added oil immersion on slide and observed at 100× lens. Selective medium xylose lysine deoxycholate agar (XLD) was prepared according to the manufacturer's protocol for the isolation of *Salmonella* sp. and same method of inoculation was used on XLD agar as was used above for the detection of *S. aureus.* The gram staining was also used for *Salmonella* sp. For further confirmation several biochemical tests were performed. Biochemical test were performed for *S. aureus* and *Salmonella* sp. by following the Begay's manual [20]. These tests were Catalase, Coagulase, Oxidase, DNase, Indole, Urease, Citrate, H_2S, Motility and Fermentation tests etc.There were three test tubes for each test; Tube A was for negative control (not containing bacteria but it contained suitable medium used for test). Tube B was for the Positive control, while tube C was containing the sample of bacterium.

2.3. Moelcular Identification

DNA was extracted by using Qiagen kit according to the manufacturer's protocol. The quality and quantity of extracted DNA was determined using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted to a working concentration of 50 ng/μL. The eluted DNA was stored at −20˚C and used for molecular identification of the isolates. The extracted DNA samples were visualized by performing gel electrophoresis. PCR was performed by using 16SrRNA primers, Forward prmer: 5' GTAGGTGGCAAGCGTTACC 3', Reverse Primer: 5' CGCACATCAGCGTCAG 3' for the detection of *S. aureus*. PCR cycling conditions utilized were, initial denaturation of 94˚C for 5 min; followed by 30 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 60 seconds and a final elongation step of 72˚C for 5 min. The PCR products were stored at 4˚C and later separated by agarose gel electrophoresis. Then *Salmonella* sp. was identified using 16SrRNA primers F 5'-GGAACTGAGACACGGTCCAG-3' and R-5'-CCAGGTAAGGTTCTTCGCGT-3'.

PCR reaction volume used for the detection of *Salmonella* sp. was set to be 25 μL. It contained 3 μL (30 ng) of genomic DNA, 1.5 μL (15 pmol) of each forward and reverse primer, 12.5 μL of 2X Master Mix and 6.5 μL of nuclease free water. Then reaction tubes were kept in the PCR machine. PCR conditions were set to be as follows: 95˚C for initial denaturation for 5 minutes, 30 cycles each 1 minute at 94˚C for denaturation, 1 minute at 60˚C for annealing and 30 second at 72˚C for extension and final extension at 72˚C for 10 minute, after termination of PCR, the PCR product was observed by performing gel electro-

phoresis.

2.4. Sequencing and Sequence Analysis

The amplified 16S rRNA gene fragment was purified and sequenced using DNA sequencing services (Macrogen, Korea). 16S rRNA sequences were analyzed by using Basic local alignment search tool (BLAST) available from the website of National Center for Biotechnology information (https://blast.ncbi.nlm.nih.gov) to identify the identical matches with existing characterized reference sequences.

3. RESULT AND DISCUSSION

S. aureus was detected in 45% samples of raw milk collected from different cities of Pakistan while 43% samples of raw milk were positive for *Salmonella* sp. (**Figure 1**). *S. aureus* revealed smooth, round and translucent colonies in nutrient agar plates. The selective medium used for the detection of *S. aureus* was Mannitol salt agar; it produced round, yellow and pinkish colored colonies (**Figure 2** & **Figure 3**). Furthermore *S. aureus* depicted gram positive character on gram staining (**Figure 4**). It exhibited purple

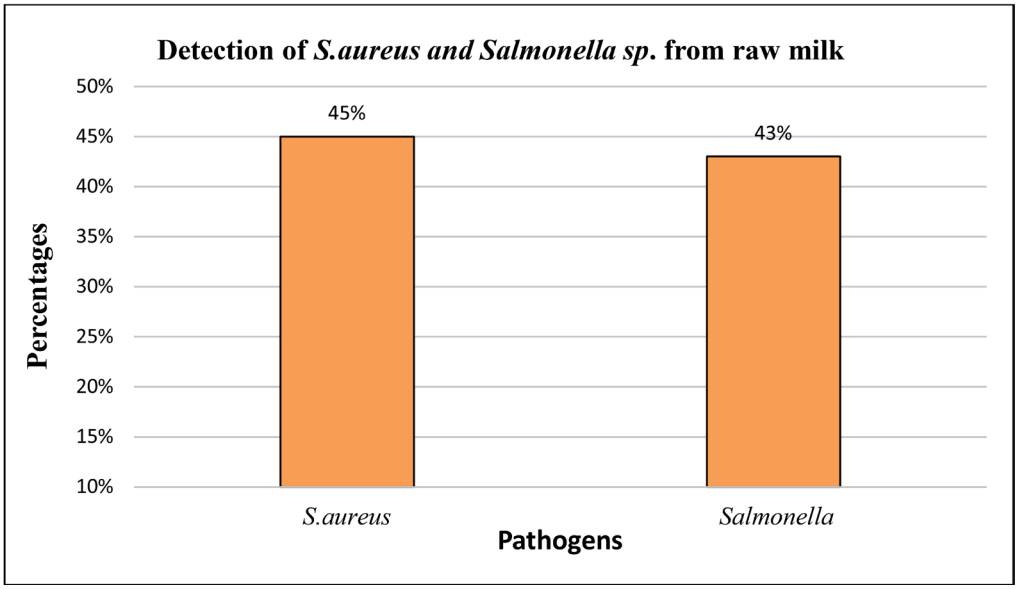

Figure 1. Detection of *S. aureus and Salmonella species* from raw milk samples of different cities of Pakistan.

Figure 2. *S. aureus* detected on Mannitol salt agar indicated by yellow and round shaped colonies.

coloration with clusters appeared on microscopic examination.

Different bacteria produce different types of enzymes such as *S. aureus* produces catalase which breakdown hydrogen peroxide and converts it into water and oxygen, hence catalase and some other biochemical tests were used for the preliminary identification of *S. aureus* and *Salmonella* spp.

Catalase test was positive for *S. aureus* in the form of bubbles of oxygen gas when Hydrogen peroxide was added in a test tube containing solution inoculated with *S. aureus* (Figure 5, Table 1).

S. aureus also produces coagulases that convert protein fibrinogen and fibrin (Figure 6). *S. aureus*

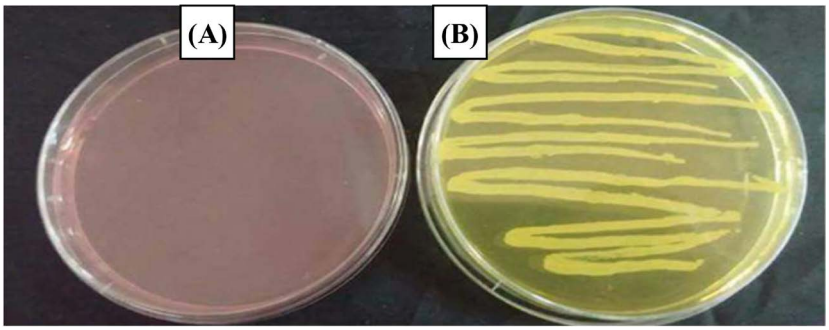

Figure 3. Colonies of *S. aureus* on Agar plate compared with a controlled sample. Detection of *S. aureus* in raw milk samples by using Mannitol salt agar medium. Petri-plate (A) shows no bacterial growth on a controlled sample where no milk sample was added. Petri-plate (B) shows the detection of *S. aureus* on Agar plate.

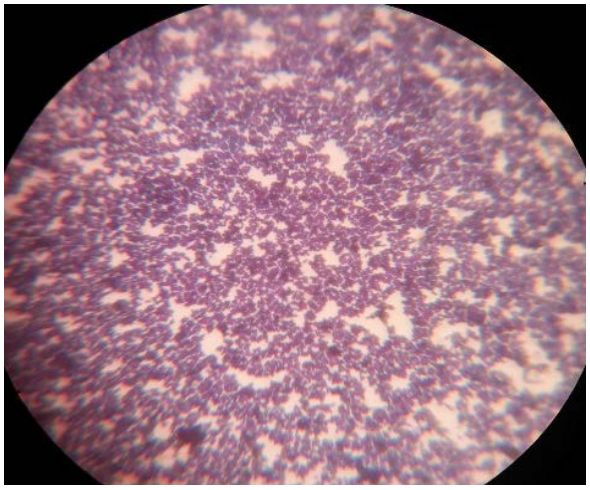

Figure 4. Gram positive character exhibited by *S. aureus*, purple colored round shaped arranged in clusters.

Figure 5. Catalase positive test exhibited by *S. aureus*.

Table 1. Biochemical characterization of *S. aureus and S. typhi.*

S. No.	Biochemical tests	*S. aureus*	*S. typhi*
1	Oxidase	−	−
2	Catalase	+	+
3	Coagulase	+	×
4	DNase	+	−
5	Indole	−	−
6	Urease	+	−
7	Citrate	+	+
8	H_2S	−	+
9	Suc. Ferm.	+	+
10	Fruc. Ferm.	+	+
11	Gluc. Ferm.	+	+
12	Mannitol	+	+
13	Motility test	Non Motile	Motile
14	Morphology Gram staining	Gram positive	Gram negative Rods

Figure 6. Detection of *S. aureus* by positive coagulase test in many samples of raw milk.

also has ability to use DNase as carbon energy source for growth. It breaks down DNA into smaller fragments, hence DNase test is also used for the identification of *S. aureus*.

Salmonella showed circular, smooth and translucent colonies on nutrient agar plates. The Xylose Lysine Deoxycholate agar (XLD) is a selective medium used for the detection of *Salmonella*. It produced round, yellow and black colonies (Figure 7). A thin smear was prepared with the colony of Xylose Lysine Deoxycholate agar (XLD) for gram staining. *Salmonella* revealed gram negative character. Pink coloration and rod shaped structure of *Salmonella* s observed when gram stained samples were observed under the microscope (Figure 8). For further confirmation of the presence of *Salmonella* in raw milk samples, bio-

chemical tests were performed. *Salmonella* also produces catalase like *S. aureus*, hence 43% samples were Catalase positive with gram negative character (Table 1). Different other biochemical tests are also used for the identification of *Salmonella* like Citritase which breaks down citrate to oxaloacetate and acetate. *Salmonella* exhibited result for citrate test positive in the form of blue coloration (Figure 9, Table 1). Urease (Figure 10), Oxidase and Indole tests were negative for *Salmonella* because these enzymes are not produced by this bacterium. Fermentation test was also found positive for *Salmonella*. *Salmonella* also has the ability to ferment different Carbohydrates like Maltose, Dextrose etc (Table 1).

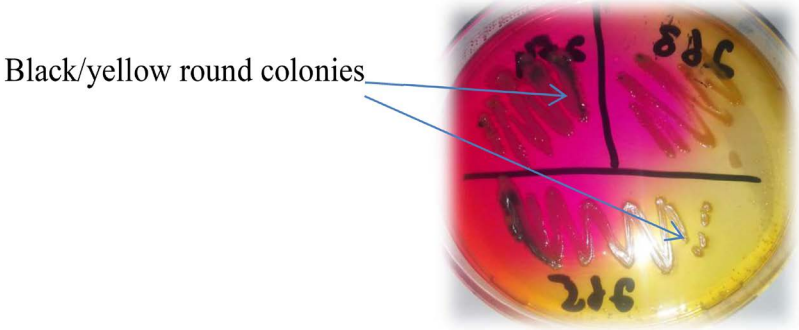

Figure 7. ***Slomonella*** **is present on Xylose Lysine Deoxycholate agar (XLD).**

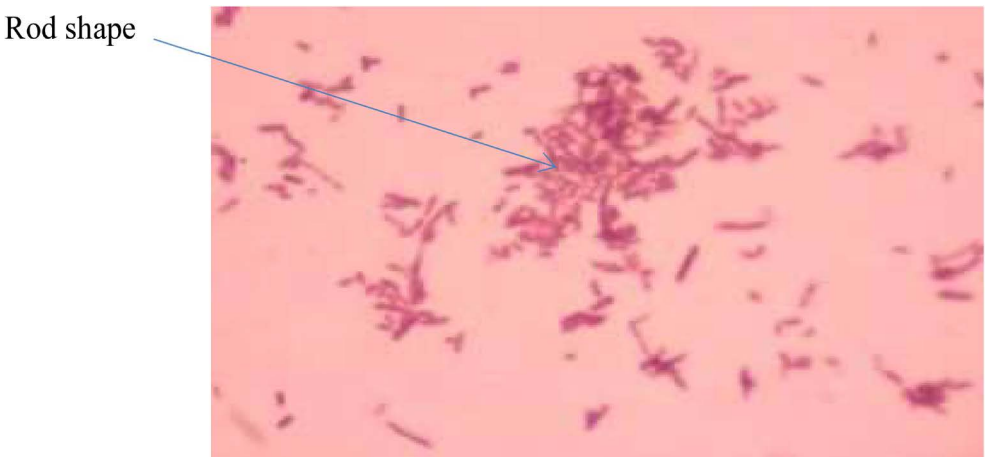

Figure 8. **Rod shaped structure of** ***Salmonella*** **under microscope.**

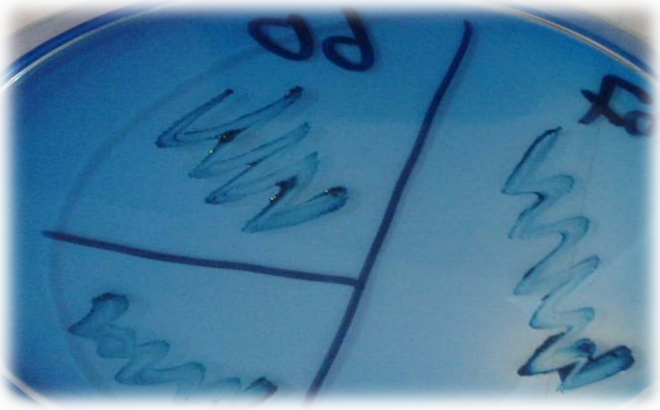

Figure 9. ***Salmonella*** **exhibited positive citrate test.**

S. aureus was detected in 91 (45%), while *Salmonella* sp. was identified in 87 (43%) out of 200 raw milk samples. The 16SrRNA sequencing confirmed the results of Biochemical analysis. A representative bacterial isolate S14 (depicting positive biochemical, cultural and morphological tests for *S. aureus*) out of 91 identified isolates was selected for 16SrRNA sequencing analysis. Samples were sent to Korea, Macrogen for sequencing. The Sequence of bacterial isolate S14 exhibited 99% identity with *Staphylococcus aureus* (Table 2) while isolate S22 (showing biochemical, cultural and morphological characters for *Salmonella* spp.) sequence exhibited 98% identity with *Salmonella* sp. (Table 2).

Accurate identification of food-borne pathogens is very important to develop the eradication techniques. Approach of using cultural, biochemical and 16SrRNA sequence analysis techniques are useful for identification of these pathogens by using conventional procedures [21, 22]. In this study, we collected a total of 200 (cow and buffaloes) raw milk samples from different cities of Pakistan. 45% of raw milk samples were positive for the presence of *staphylococcus aureus* while 43% samples exhibited the presence of *Salmonella* sp. Food borne diseases are very common due to the presence of these pathogens in developing countries. Previous studies are in agreement of our findings of the detection of pathogens in raw milk [12-14]. The percentages of *S. aureus and Salmonella* sp. is higher in our findings as compared to the previous literature. This difference in prevalence of these pathogenic bacteria in unpasteurized milk samples might be due to difference of geograophical location, diagnostic approaches used for the detection etc. Several other pathogens are also detected in other studies. Mycobacterium spp. was detected from 8% Bovine milk samples in Brazil [23]. Listeria monocytogenes is another agent of food borne diseases (3). These pathogenic microoraganisms produce extracellular protein toxins which are considered to be responsible for the pathogenicity of the organsims. Contamination may be added in milk by different sources for example by environment, handling, milk container, milk handler, utensils and storage, dirty udders of cows and buffaloes etc. Some diseased animals secrete pathogenic bacteria in their milk and when this milk is consumed by humans, it causes toxic effects. Due to the lack of cooling facility, microorganisms multiply in it and then cause different types of diseases. These bacteria cause several diseases in humans who consume raw milk without heating or boiling. People should avoid the consumption of milk without boiling

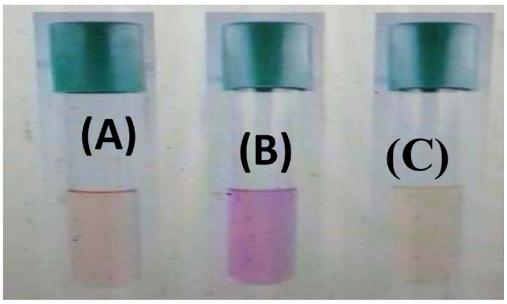

Figure 10. *Salmonella* exhibited Urease test negative as depicted in figure by sample C. Tube (A) shows an un-inoculated medium while Tube (B) shows positive control with *Proteus vulgaris* added.

Table 2. Moelcular identification of unpasteurized milk pathogens.

Primer	Sequence	Identified Microoragnsism	Amplicon Size (bp)
16S rRNA F	5'-GTAGGTGGCAAGCGTTACC-3'	*S. aureus*	228 bp
16S rRNA R	5'-CACATATGTTCTTCCCTAATAA-3'		
16S rRNA F	5'-GGAACTGAGACACGGTCCAG -3'	*Salmonella* sp.	660 bp
16S rRNA R	5'-CCAGGTAAGGTTCTTCGCGT-3'		

and should adopt proper hygienic measures in handling of raw milk and its products. The higher percentage of *S. aureus* and *Salmonella* in present research is in accordance with the results of many researchers eg., Donkor *et al.*, (2007), who revealed a similar study on raw milk samples of Accra and Kumasi cities. They cultured and identified different pathogenic bacterial strains in unpasteurized milk [24]. They demonstrated that due to poor hygiene condition probable fecal contamination of the milk was mostly caused. Another source of contamination by microorganisms is unclean teats. The use of unclean milk transport equipments may also contribute to the poor hygienic quality [25]. Present results are also in agreement with Badini *et al.*, (1996) who reported the contamination of 50% samples of raw milk with *Staphylococcus aureus* [26]. Another report also demonstrated the presence of pathogenic bacteria in unpasteurozed milk. They demonstrated the presence of toxin producing *Staphylococcus aureus* in 77% of unpasteurized milk samples [27, 28].

4. CONCLUSION

The raw milk available to consumers in Pakistan (Feb 2015-June 2015) was contaminated with pathogenic bacteria like *S. aureus* and *Salmonella.* Different other pathogenic micro-organisms may also be present in raw milk samples. This might be due to unhygienic condition during processing, storage and handling of unpasteurized milk. It could also be due to the use of raw milk from diseased animals. There may be *S. aureus* and *Salmonella* sp. infections among cows and buffaloes. Investigation studies of pathogens in unpasteurized milk will facilitate the evaluation of safety of raw milk products and will help in the development of novel processing technologies. Antibiotic resistance of pathogenic bacteria isolated from milk may be tested further to analyze the severity of these pathogenic microbes. Non culture based identification of bacteria in milk can also be performed by protein finger printing. The use of protein markers for non-culture-based bacterial identification allows for high-throughput detection of pathogens present in milk sample.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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