Annals of R.S.C.B., ISSN:1583-6258, Vol. 26, Issue 1, 2022, Pages. 364- 374
Received 08 November 2021; Accepted 15 December 2021.

Antimicrobial Resistant and Sensitivity Profile of Bacteria isolated From Raw Milk in Peshawar, KPK, Pakistan

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ABSTRACT

The extensive progress of dairy sectors in developing countries like Pakistan led to widespread use of antibiotics to improve the health and productivity of animals; prolonged usage may lead to antibiotics residues in foods of animal origin: hence, the emergence of antimicrobial-resistant microorganisms. Accurate data on antibiotic use in livestock treatment, antibiotic residues and antimicrobial resistances in raw Milk in Pakistan are lacking. The study's main objective was to investigate the susceptibility profile of bacterial isolates from raw milk used for drinking and other purposes within different 5 areas of the Peshawar city and to create awareness among the people about pasteurization and sterilization of raw milk. A total of 12 raw milk samples were evaluated. The bacterial isolates were identified and measured of resistances to 10 antibiotics most commonly used during bacterial infection. Amongst all 4 (33.34%) isolates were positive to *S. aureus,* 3 (25%) isolates were positive to *E.coli,* 3 (25%) were positive to *Pseudomonas sp,* and only 2 (16.66%) isolates were positive to *Bacillus sp.* Determination of the antibiotic resistance pattern of isolates showed that among the Gram-positive bacteria, *S. aureus* and *Bacillus sp*. They were highly susceptible to Augmentine, Erythromycin, Ampicillin, Gentamycin and resistance to clindamycin and ceftriaxone. While among the Gram-negative bacteria, *E.coli* displayed significant resistance to Levofloxacin, Vancomycin, Amoxicillin, Gentamycin. At the same time, *E.coli* were susceptible to Tetracycline, Ciprofloxacin. *Pseudomonas aeruginosa* showed resistance to Vancomycin, Oxacillin, and Amoxicillin while sensitive to Ciprofloxacin and Tetracycline.

Keywords: Antibiotics, Peshawar, raw milk, microorganism, Antimicrobial activity

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1. INTRODUCTION

Milk is the natural secretion of the mammary glands of mammals. It contains a large variety of nutrients more than other foodstuffs. It is a raw food for young and adults, especially cows and goats. Other mammals that produce milk for human consumption are buffalo, sheep, goats and camels. The milk of different animal species contains the same constituents but varies in composition (1, 2). Milk is a completely balanced diet with the right amount of carbohydrates, protein, fats, vitamins and minerals. Bacteria of sorts thrive in milk and, as a result, reduce its quality. The presence of pathogenic bacteria in milk is of immense public health significance. The hands of unhygienic milk handlers, the housing environments and instruments, and the cow itself are possible sources of milk contamination by pathogenic bacteria (3). Cow's milk is a predominant and pale liquid produced by cow's mammary glands. It is the primary source of nutrition for infant mammals before they can digest other types of food (4). It contains many other nutrients, including protein and lactose, but some depend on goat milk, where the goats are kept by the families and camel milk by nomadic people. Internationally, sheep are reared mainly for their meat and hide, not for Milk (5).

Milk is considered an excellent medium for the growth of many microorganisms. Milk can be contaminated with several bacteria during the milking process from the milking personnel and utensils used from milking animals (6). Besides, microorganisms may enter the udder through the teat canal, and the bacteria may come out through Milk (7). *Staphylococcus aureus* and *Escherichia coli* are the two major contaminants of milk. The presence of the pathogens in milk largely depends on faecal contamination, and the presence of the pathogen in faeces mainly originates from feed contamination (8). Good quality milk meets the body's nutritional needs better than any single food as it contains essential food constituents such as fat, proteins, carbohydrates, minerals, and vitamins (9). As a result of these nutritional components, milk is an excellent culture medium for many microorganisms, especially bacterial pathogens (10). Foodborne diseases are of great concern around the world. However, this is an important issue in developing countries where poor sanitation is maintained during the collection and processing of milk from cattle and buffaloes (11). *Staphylococcus aureus* is an important pathogen for dairy ruminants causing inflammatory reactions, and the organism is believed to cause 30-40% mastitis (12, 13). The organism can be excreted directly from the udder through Milk (14). *S. aureus* milk indicates the hygienic standard followed during the milking process. Information on antibiotic resistance against *S. aureus* could be useful in treating the disease caused by these organisms (15). *Eschereci*a is one of the important bacteria of gut flora .Among the pathogenic *E. coli*, Shiga toxigenic *E. coli* (STEC) strains have been reported mostly in Latin America, India, Bangladesh and many other developing countries. Pathogenic *E. coli* have been isolated by several researchers in Bangladesh from faecal samples of healthy cattle's and buffalo's raw Milk (15, 16).

To extend the shelf life of milk for human consumption and prevent the growth of spoilage causing microorganisms and prevent transmission of diseases via milk, this highly nutritious, versatile food is usually pasteurized. Unfortunately, many workers have reported post pasteurization

contamination of milk with resistant pathogenic (17). For instance, some potential human pathogens, such as *Mycobacterium tuberculosis*, *Bacillus cereus*, *Clostridium spp., Listeria monocytogenes* and *Salmonella spp.,* have been reported to survive conventional heat pasteurization in Milk (18). To produce the milk of good hygienic quality, it is important to have clean healthy cows and clean utensils for milking and to store the milk. Unfortunately, the consumption of unpasteurized milk in most developing countries, including Nigeria, has not attracted the desired attention. Bacteria are widely distributed in nature and may be introduced into milk easily. Consequently,abroad spectrum of bacteria such as *Staphylococcus aureus, Escherichia coli, Salmonella spp., Pseudomonas spp., Enterobacter spp., Klebsiella spp., Proteus spp.* and *Yersinia spp,* have been recovered from raw milk, and some of these have been determined to be potentially pathogenic and toxicogenic and implicated in milk-borne gastroenteritis (18). The main objective of the study was to investigate the susceptibility profile of bacterial isolates from raw milk that is used for drinking and other purposes.

2. MATERIALS AND METHOD

2.1. Sample collection

Twelve raw milk samples were collected from five different places of Peshawar city, i.e. Kohat road, Bara road, Laundi Akhun Ahmad (SUIT area), Sadder and Hayatabbad. About 100 ml of milk sample was taken milk stored in a container in blue cap bottles aseptically. All the samples were transported using a sample collector icebox at 4°C to Microbiology Laboratory and were incubated at 37^0C for 2 hours.

2.2. Sterilization of glassware and other material

All glassware used was thoroughly washed with detergent, rinsed and allowed to dry. The glassware was then wrapped with aluminium foil and sterilized in an autoclave for 15 min at 121. The distilled water used for serial dilutions was also autoclaved at 121°C for 15 min. The workbench was swabbed with 70% ethanol before and after every experiment.

2.3. Serial dilution

A test tube added one ml sample milk taken from each blue cap bottle through micropipette to 9 ml of sterile and autoclaved distilled water. It was serially diluted up to 10^{-10}. Then 1 ml was aseptically collected from each dilution of 10^{-3} and 10^{-10} and poured onto the sterile nutrient agar plates using the spread plate technique. The same procedure was repeated with all the samples. Finally, these plates were inverted and incubated at 37°C for 24 hours in the incubator.

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2.4. Pure culture

For obtaining a pure culture and clear morphology, subculture was performed on Eosin Methylene Blue Agar (EMB), MacConkey Agar (MCA), and Mannitol Salt Agar (MSA) plates and then incubated at 37°C for 24 hours. The same procedure was performed on fresh media for obtaining pure culture.

2.5. Morphological and biochemical identification of Bacteria

The isolated bacteria were examined by gram's staining test to differentiate between gram-positive and gram-negative bacteria and their morphology. Further identification of bacteria was made by performing a series of biochemical tests using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology such as Coagulase, Catalase, Triple Sugar Iron and Indole test were performed.

2.6. Study of Antibiogram

The disk diffusion method was used for antimicrobial resistance and susceptibility using CLSI guidelines. Impregnated antimicrobial disks were placed on inoculated Muller Hinton agar (MHA) media and placed in the incubator for 24 hours at 37°C. The antibiotic tested disk were Ciprofloxacin(10 µg), Tetracycline(30 µg), Levofloxacin (20 µg), Amoxicillin(30 µg), Oxacillin (10 µg), Gentamicin(30 µg), Augmentin (10 µg), Ceftriaxone (20 µg), Ampicillin(10 µg), Cotrimoxazole(10 µg), Erythromycin(30 µg), Vancomycin (10 µg) and Clindamycin(10 µg). The diameters of the inhibition zone around the disks were measured using a Vernier calliper. The results were recorded as sensitive, resistant, and intermediate.

3. RESULTS

3.1. Isolation of bacteria from raw milk samples

A total of 12 bacterial species were isolated from raw milk samples distributed among two Gram-positive genera *Staphylococcus*, *Bacillus* and two-gram negative genera. *Escherichia coli*, *Pseudomonas.*To observe the morphological characteristics of isolated species, gram staining was done. Table 1shows the percentage presence of isolated bacteria.

Table 1. Percentage of bacterial load of milk samples.

S. No.	Isolates	Gram reaction	No. of isolates	Percentage
1.	*Bacillus sp.*	Positive	4	16.66%
2.	*Staphylococcus aureus*	Positive	2	33.34%
3.	*Escherichia coli*	Negative	3	25.00%
4.	*Pseudomonas sp.*	Negative	3	25.00%
TOTAL			**12**	**100%**

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3.2. Identification of bacterial species through biochemical tests

Twelve bacterial isolates were isolated from the raw milk samples. All these 12 isolates were identified and characterized through different Biochemical tests (Catalase test, Coagulase test, Indole test and TSI). These isolates were identified as *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Bacillus sp.* (Table 2).

Table 2. Biochemical characteristics of bacterial isolates from raw milk.

S. No.	Biochemical test	Gram-Positive Bacteria		Gram-negative Bacteria	
		Staphylococcus aureus	***Bacillus sp.***	***Pseudomonas sp.***	***E. coli***
1	Catalase	Positive (+)	Positive (+)	Negative (-)	Negative (-)
2	Coagulase	Positive (+)	Positive (+)	Negative (-)	Negative (-)
3	TSI	A/A H_2S	K/K	K/A H_2S	K/K
4	Indole	Negative (-)	Positive (+)	Positive (+)	Positive (+)

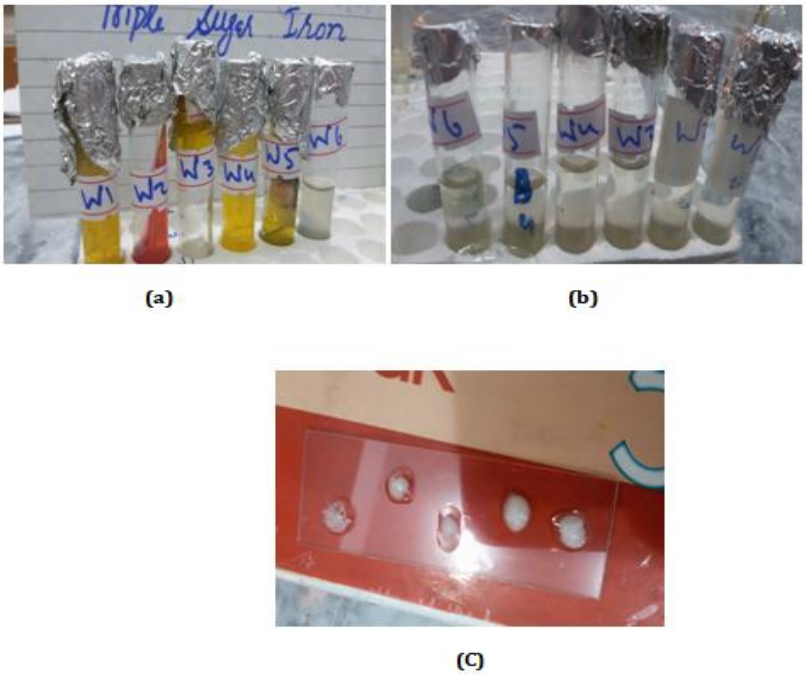

Figure 1. Showing (a)Triple sugar iron, (b) Indole test and (c) Catalase test for bacterial isolates

3.3. Antibiotic susceptibility profile

Table 3and Figs. 3 show the antibiotic susceptibility patterns of each species. Among the Gram-positive bacteria, *Staphylococcus* and *Bacillus sp.* were highly susceptible to Augmentin, Erythromycin, Ampicillin, Gentamicin and resistant to clindamycin and ceftriaxone.

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Table 3. Antibiotic susceptibility patterns of gram-positive bacterial isolates

S.No	Antibiotic	Concentration (µg)	Mean Diameter of Inhibition Zone (in mm)	
			S. aureus	***Bacillus sp.***
1	Augmentin	10	22(S)	26(S)
2	Ceftriaxone	20	13(R)	15(I)
3	Ampicillin	10	21 (S)	23(S)
4	Cotrimoxazole	10	20(S)	25 (S)
5	Erythromycin	30	20 (S)	15(I)
6	Gentamicin	30	19 (S)	20(S)
7	Clindamycin	10	16 (I)	14(I)

I = Intermediate sensitivity, S=Sensitive, R=Resistant, Zone of Inhibition: 0-13 mm = resistance; 14 -18 mm = Intermediate sensitivity; 18 mm and above = Sensitivity

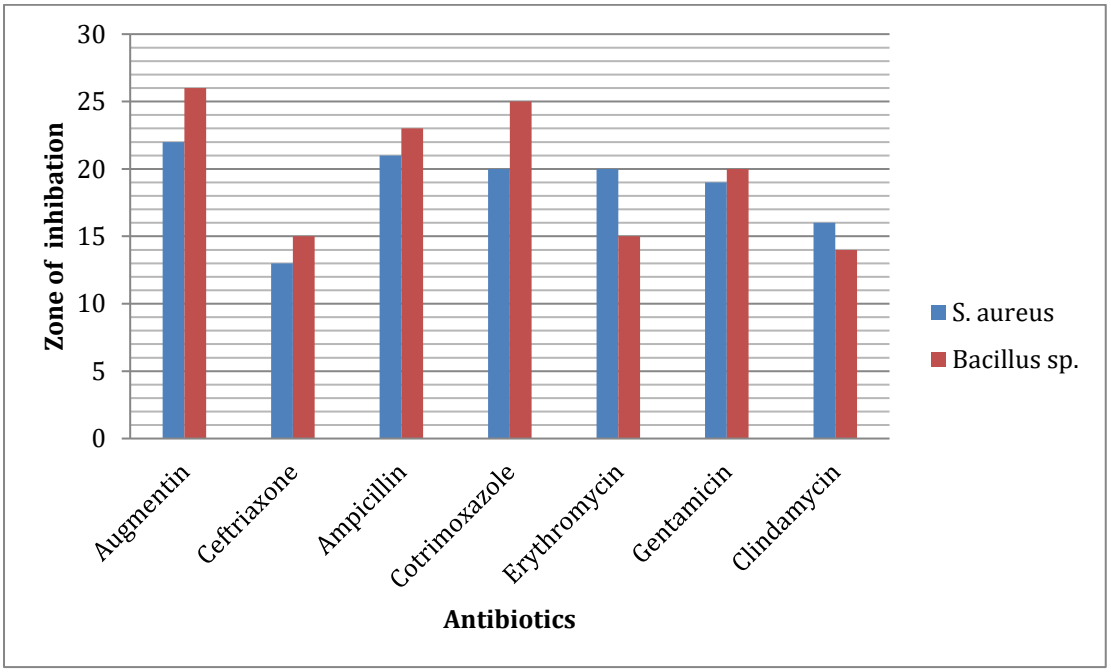

Figure 2. Antibiotic susceptibility and resistance patterns of gram-positive bacterial isolates

Among the Gram-negative bacteria, *E.coli* displayed significant resistance to Levofloxacin, Vancomycin, Amoxicillin, Gentamycin. At the same time, *E. coli* were susceptible to Tetracycline, Ciprofloxacin. *Pseudomonas aeruginou*s showed resistance to Vancomycin, Oxacillin, Amoxicillinwhile being found sensitive to ciprofloxacin and Tetracycline. All these findings are shown in Table 4.

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Table 4. Antibiotic susceptibility patterns of gram-negative bacterial isolates

S.No	Antibiotic	Concentration (µg)	Mean Diameter of Inhibition Zone (in mm)	
			Pseudomonas sp.	***E. coli***
1	Levofloxacin	20	16 (I)	7 (R)
2	Vancomycin	10	8 (R)	11(R)
3	Oxacillin	10	9 (R)	14(I)
4	Amoxicillin	30	6 (R)	8 (R)
5	Tetracycline	10	19 (S)	21(S)
6	Ciprofloxacin	10	19 (S)	19 (S)
7	Gentamicin	30	16 (I)	8 (R)

I = Intermediate sensitivity, S=Sensitive, R=Resistant, Zone of Inhibition: 0-13 mm = resistance; 14 -18 mm = Intermediate sensitivity; 18 mm and above = Sensitivity

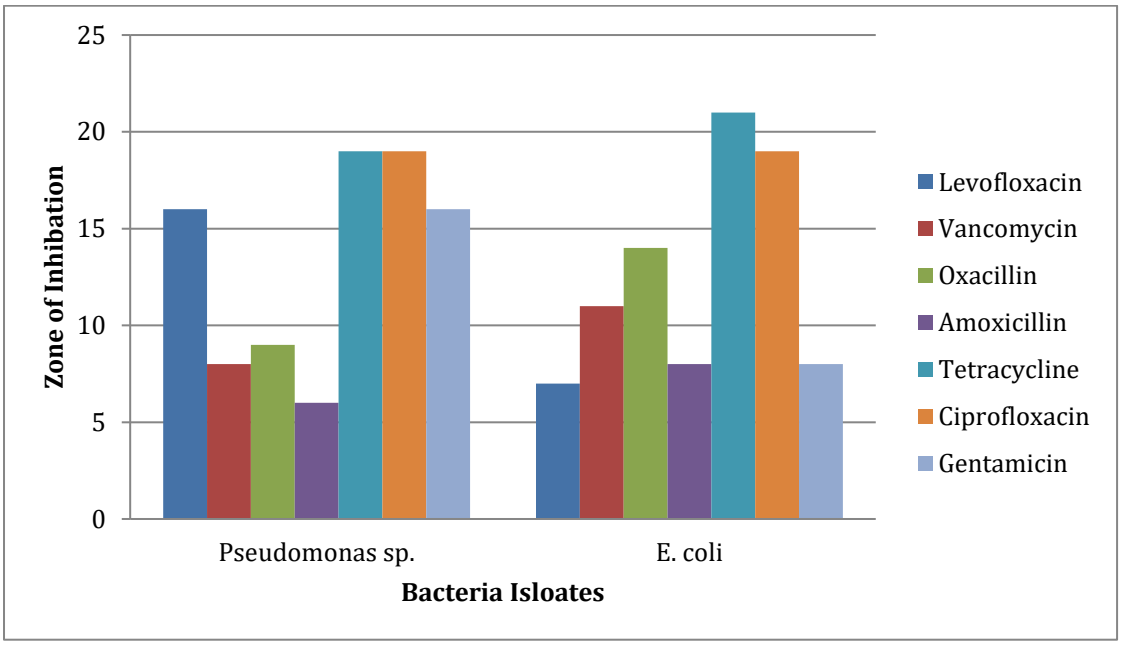

Figure 3. Antibiotic susceptibility and Resistance patterns of gram-negative bacterial isolates

4. DISCUSSION

Antibiotic susceptibility is important in monitoring food borne pathogens for their effective control in the herd and preservation of dairy products. Several animal pathogens can cause human disease, and they are well known to be transmitted to humans through the consumption of raw milk. The high numbers of isolated microorganisms contaminate the milk and multiply and grow in the available media. This is because milk is a good nutritive medium for the growth of microorganisms due to the impact of poor sanitary procedures and lack of appropriate cooling facilities (19). In the current study, 5 milk samples collected from different areas of Peshawar city were analyzed. A total of 12 bacterial species were isolated from raw milk samples distributed among two gram-positive genera. *e. Staphylococcus, Bacillus* and two gram-negative genera, i.e.*Escherichia coli*,

Pseudomonas. A total of 12 potential isolates were sub-cultured and further analyzed. All the 12 isolates satisfied the identification criteria and were used for subsequent analysis. All the 12 bacterial isolates were subjected to antibiotic susceptibility tests. In the current study, *E. coli* was observed (25%) being in agreement with the data obtained by (20) that observed *E. coli* prevalence (12.9%) in buffalo milk samples from Punjab, India. Moreover, Kumar (2009) observed buffalo milk samples with a high mastitis *E. coli* (30%). Both the studies are in great compliance with the current research. The incidence of *Bacillus spp*. Observed (16.66%) is a similar study reported (21, 22). Among the gram-positive bacteria, *Staphylococcus* and *Bacillus sp.* were highly susceptible to Amoxicilline, Erythromycin, Ampicillin, Gentamicin and resistant to clindamycin ceftriaxone. Reported erythromycin and Tetracycline as effective antibiotics against *Staphylococcus* and *Bacillus sp.*(23) also reported similar findings against *Staphylococcus aureus* isolated when large erythromycin doses were used. The present study results are identical to those of the above workers. Moreover, when low doses of antibiotics are used against bacteria, they inhibit the growth of susceptible bacteria and leave a smaller number of already resistant bacteria, which thrive and grow. These bacteria spread their resistance traits to other previously non-resistant cells than eventually affecting other cells (24). The majority of the gram-negative cultures showed resistance to the third generation viz. Levofloxacin, Vancomycin, Amoxicillin, Gentamycin and Oxacillin are in concordance with the most recent reports of drug resistance patterns in investigations conducted on raw milk pathogens (25, 26). Thus, the possible long-term indiscriminate use of these antibiotics in the region has led to the appearance of resistance in bacteria against third-generation antibiotics (27, 28). The highest drug resistance recorded in the current study might be due to high antimicrobial use in dairy farms and individual cows to treat various diseases affecting the dairy sector. Antimicrobial resistance emerges from using antimicrobials in animals and humans and the subsequent transfer of resistance genes and bacteria among animals, humans, animal products, and the environment.The results of this study are in line with the findings of other studies conducted in different parts of the (29). However, antimicrobial resistance rates obtained in this study were higher as compared to susceptibility patterns reported from previous studies (30)The remarkable degree of resistance to many drugs represents public health hazard because foodborne outbreaks would be difficult to treat. This pool of MDR *E. coli* in the food supply represents a reservoir for communicable resistant genes. Hence, due to the relatively limited access and high price to get the newly developed cephalosporin and quinolone drugs, the reports of the prevalence of antimicrobial-resistant bacterial isolates from milk to relatively low-priced and regularly available antibiotics are alarming for a low-income society living in most developing countries, like Pakistan. The present study shows that more efforts are needed to enhance and promote farms and sale points of milk by following confirmatory tests to check the microbial quality of the milk. Moreover, the concerned ministries should adopt a comprehensive strategy for ensuring a safe supply of good quality milk. These strategies should include promoting the knowledge of farmer's standards through training, extension programs, adoption of grading and quality testing of milk. Ultimately, the milk testing programs should become components of the quality process that

should focus on producing high-quality milk, not only at the preservation and supply level, but also at the production herd level.

5. CONCLUSIONS

The current study gives us a redirection to the sensitivity profiles of *S. aureus, Puesodomonas, E Coli and Bacillus* isolated from milk samples against the mentioned commonly used antibiotics in Suit Microbiology lab and also depict the multi-drug resistance of the same bacterial isolates in a variety of milk samples which ultimately give a point to indiscriminate use of antibiotics. And it is a matter of great concern for human health because we are the people who consume these animal products. Moreover, it is concluded that the microbiological quality of most of the raw milk samples collected from different areas of Peshawar city was not satisfactory as some pathogenic bacteria such as *E. coli*, *Puesodomonas*spp. and *Staphylococcus* spp. were detected from the samples. The presence of *S.aureus*and *Bacillus* spp. will render milk unfit for human consumption since many of these organisms will cause infection and intoxication. Multiplication and production of *S. aureus* depend on environmental factors like time, temperature, relative humidity and duration of storage and food factors, potential water activity and moisture content present. These multi-drug resistant bacteria may no longer be treated with conventional therapeutic drugs, and they are also capable of spreading their resistant gene to other bacterial genera. So, frequent use of antibiotics should be prohibited. And the government should be concerned about that. It was further concluded that all the raw milk sources in Peshawar city are poor microbial quality. It is recommended that a possible follow-up be made to identify the sources of contamination. It is also recommended that the milk storage utensils be washed out properly before using the milk for taking or cooking purposes.

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