TIMES JOURNAL OF HEALTH AND SOCIAL SCIENCES
Original Article

PRESENT STATUS OF MICROBIOLOGICAL EVALUATION OF RAW MILK IN MULTAN CITY

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Abstract

Raw milk can oblige as energetic basis of nourishment nonetheless it can act as potential standard for conveying infective microorganisms and cause transferrable sicknesses. This study evaluates contagious superiority and antimicrobic confrontation of microorganisms secluded from raw milk advertised in Multan city, Pakistan. A whole of 300 raw milk tasters was together from several spaces. Standard microbiological method was employed for bacterial identification and antibiotic vulnerability testing was achieved by means of disc diffusion procedure.

The outcomes exposed that mainstream of milk tasters were dirtied by pathogenic microbes, including *Escherichia coli* (66%), *Staphylococcus aureus* (45%), *Shigella* (14%), *Proteus* (19.6%), *Listeria* (16.3%) and *Salmonella* (8.6%). The pH of most samples was slightly acidic which suggest poor handling. On the other hand, antimicrobial defenselessness testing exhibited tall confrontation to ampicillin and doxycycline while ciprofloxacin and cefixime were the most effective antibiotics. Uncovering of Multidrug Resistant (MDR) isolates particularly amid *E. coli* besides *S. aureus*, shows the emerging treat of antimicrobial resistance in dairy sector.

This study concludes that poor hygiene during milking, management then conveyance significantly contributes towards milk adulteration. To ensure microbial milk safety in Pakistan, government should strengthen food safety regulation and promote hygienic practices.

Keywords: Raw milk, Microbial contamination, antimicrobial resistance, Multan, Pakistan, Food safety.

1. Introduction

All over the world, milk is a major component of human diet. It contains higher nutrient values especially for children which helps them in their growth phase. Due to the higher nutrient contain, milk can also be a great medium for microorganism growth. Due to low hygienic conditions, number of pathogenic bacteria can easily contaminate it. Thus, the quality of milk should be very important for the community (21).

In Pakistan, additional eighter squillion peoples are part of livestock manufacture. Over the years, livestock sector has surpassed the crop production as the biggest contributor of the agriculture sector. Livestock production has contributed 11.7% of the overall GDP of Pakistan. Gross value addition in livestock has increased from 1430 billion (2018-19) to 1466 billion rupees (2019-20) (10). It has been estimated that during this period, about 49.60 million cattle, 41.2 million buffalo, 31.20 million sheep, 78.20 million goats and 1.1 million camels were maintained in the country producing a total estimated milk of about 61.69 million metric tons of milk, out of which 49.737 million metric tons was estimated to be available for human consumption that has 6.1 per cent share in the GDP (7).

Pakistan is ace of major milk manufacturing country in biosphere, while remaining milk deficient. The quality of milk supplied to the household is not up to the world health standards. The 92% of the total milk supplied in the market is untreated. The unpacked milk is mostly contaminated due to less hygienic conditions which make it hazardous to use and causes spread of multiple diseases like tuberculosis (19,21). Fresh milk drawn from healthy cows normally contains a low microbial count but it can increase up to 100 fold during storage (15). Contamination can occur through infected animals, unhygienic milking practices, water supply, equipment and handlers (13). Common pathogens associated with milk contamination include *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Campylobacter, Salmonella and Yersinia* spp. (16, 8). Occurrence and development of such germs lessen milk superiority, diminution its ledge lifespan and contemporary jeopardies to mutually civic healthiness then budget (13, 11).

Milk delivers perfect atmosphere for pathological evolution due to its tall aquatic gratified, unbiassed pH and amusing nutritious configuration (24). Bacteriological uncleanness can befall from unsanitary management then storing. Deprived treatment besides derisory cooling subsidizes to putrefaction and upsurge jeopardies of milk borne ailments such TB, diphtheria then undulant fever (21). Pathogenic creatures such as *Salmonella spp*., *Bacillus cereus* besides *Clostridium botulinum* can source of diet intoxication besides further sicknesses (13).

Owing to nourishing also monetary imperative of milk then high peril of infection, it's compulsory to checked contagious eminence of raw milk (18, 19). This study was showed to appraise bacterial worth of raw milk promoted in Multan, Pakistan, with primarily emphasis on bacteriological pollution, antibiotic confrontation and related menace influences.

2. Material and Methods

2.1.Study area and Sample collection

This study was directed in Multan city, which is alienated into 185 Union Council and Civic Establishments. For specimen determination, city was separated into 5 zones: Zone 1 (UC 1-37), Zone 2 (UC 38-75), Zone 3 (UC 76-111), Zone 4 (UC 112-148) and Zone 5 (UC 149-185) as shown in Table 1. A full of three hundred raw milk tasters were placid haphazardly, with sixty models attained from apiece zone. Tasters were gotten from dissimilar foundations counting minor dairy ranchers, resident salespersons and milk assortment cores.

Respectively model (50 ml) was poised in germ-free Falcon tubes then categorized appropriately. Tasters were located in cloistered cool cases then elated to Postgraduate Microbiology Laboratory, Department of Pathobiology, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan. To minimize microbial growth, each sample was processed within same day of collection (21, 3).

Table 1 Zone formation according to Union Council and Municipal Corporation

S.No.	Zones	Location	No. of samples
1	Zone 1	UC No 1-35	60

2	Zone 2	UC No 36-74	60
3	Zone 3	UC No 75-112	60
4	Zone 4	UC No 113-146	60
5	Zone 5	UC No 147-185	60

2.2. pH determination

The pH of each milk sample was checked using a calibrated digital pH meter on the day of collection to confirm the quality of the sample (24).

2.3.Microbiological analysis

2.3.1 Total bacterial and coliform counts

Standard plate count techniques were performed according to CLSI (2023) protocols. Ten-fold serial dilutions (10^{-1} to 10^{-6}) of each milk sample were prepared using sterile peptone water as the diluent. One milliliter from each dilution was inoculated onto Nutrient Agar aimed at total bacterial count (TBC) then MacConkey Agar intended for coliform count. After inoculation, Petri dishes were gestated at 37^{o}C meant for 24 to 48 hours. Plates with 30-300 colonies were selected for enumeration (4, 1).

2.3.2. Enumeration and identification of pathogens

- S. aureus was isolated on Mannitol Salt Agar and Staph 110 medium and identified based on Gram staining, catalase test and coagulase test (8).
- Spore-forming bacteria were enumerated by heating dilutions at 80 ^{o}C for 10 minutes and plating on Starch Milk Agar for aerobic spore-formers and Clostridial Agar (RCA) for anaerobic spore-formers. Anaerobic conditions were created using anaerobic jars with palladium catalysts. Plates were incubated at 37 ^{o}C for 2-4 days (11).

- *Escherichia coli* were detected on MacConkey Agar and confirmed using Eosin Methylene Blue (EMB) Agar following ISO 7251:2005(E). Colonoies showing characteristic metallic sheen were further subjected to biochemical tests.
- *Salmonella* and *Shigella* were isolated using SS (*Salmonella Shigella* Agar and confirmed through biochemical tests including TSI (triple sugar iron) agar slants (9).

2.3.3. Biochemical tests

Isolates were subjected to confirmatory biochemical tests including lactose fermentation, indole tests, methyl red, Voges-proskauer and citrate utilization tests to confirm *E. coli* and differentiate coliforms (2).

2.4.Methylene Blue Reduction Test (MBRT)

To calculate excellence of raw milk, Methylene Blue Reduction test was accomplished. The rate of decolonization of methylene blue indicated the bacterial presence and based on that results milk samples quality was categorized as excellent, good, fair or poor quality milk (18).

2.5.Antimicrobial susceptibility testing

Antibiotic resistance profile of bacterial isolates was determined using Kirby-Bauer disc diffusion method following CLSI (2023) guidelines. The pure culture inoculum was blowout on Mueller-Hinton Agar plates and commercial antibiotic discs were applied. Plates were nurtured at 37^{o}C for 24 hours and precinct of inhibition were restrained. Tested antibiotics included Ampicillin, Doxycycline, Enroflaxicin, Ciprofloxacin, Neomycin, Gentamycin, Cefixime and Streptomycin used in veterinary and human medicine in Pakistan. *E. coli ATCC 25922* was used as a quality control organism for the antimicrobial susceptibility test (12). Insulates viewing confrontation to 3 or further antimicrobic courses were measured as MDR.

2.6.Statistical analysis

All results were recorded in Microsoft Excel (2010). Descriptive statistics including mean, median and standard deviation were calculated using RStudio (2024.12.1) (23).

3. Results

A total of 300 raw milk models were composed from five unlike sectors of Multan city besides explored of legalize their infectious outline. Findings are summarized below.

3.1.pH of milk samples

The pH of all uncooked milk samples were recorded on the day of collection. Values ranged from 6.2 to 6.8, which falls close to the normal range of fresh milk (6.6-6.8). However, minimal reduction in pH was observed in some samples. Among the five zones, the lowest mean pH was detected in Zone 4 (6.2), while Zone 1 showed the highest mean pH (6.8).

3.2.Total bacterial and coliform counts

Total bacterial counts (TBC) were obtained for all 300 samples. Considerable variation was observed among the five zones. The highest bacterial load was recorded in Zone 4, while the lowest bacterial counts were found in Zone 1. Despite these differences, the overall mean of bacterial counts in all zones were above internationally recommended limits for raw milk. The maximum and minimum values of total plate count of bacteria in milk sample from 5 different zones are shown in Table 2.

Coliform counts (CC) were also consistently high across all zones. All samples confirmed positive for coliforms. Uppermost coliform load was chronicled in Zone 3, while relatively lower were observed in Zone 5 but still unacceptable. Table 3 shows the mean total count of coliforms bacteria in milk from 5 different zone of sample source.

Table 2: Max and Min values of Total Plate count of bacteria in milk sample from 5 different zone of Multan.

Zone	Zone 1		Zone 2		Zone 3		Zone 4		Zone 5	
Sample	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**

source	Log10 cfu/ml	Log10 cfu/ml	Log10 cfu/ml	Log10 cfu/ml	Log10 cfu/ml	Log10 cfu/ml	Log10 cfu/ml	Log10 cfu/ml	Log10 cfu/ml	Log10 cfu/ml
Milk shops	6.97	5.75	6.87	5.79	6.83	5.51	6.82	6.51	6.56	4.51
Milk Men	6.93	4.88	6.85	5.80	6.75	5.65	6.75	5.62	6.66	4.62
Dairy Farm	5.86	4.62	5.70	5.40	4.68	4.36	6.66	4.34	5.66	3.34
Collection center	5.83	5.64	6.81	5.57	5.81	5.52	6.62	4.56	6.62	4.62
Milk dairies	6.86	5.73	5.83	5.51	5.78	5.66	6.66	5.36	5.86	4.66

Table 3: Mean Total Plate count of Coliforms bacteria in milk from 5 different zone of sample source.

Sample source	Zone1 Log10 cfu/ml	Zone2 Log10 cfu/ml	Zone3 Log10 cfu/ml	Zone4 Log10 cfu/ml	Zone5 Log10 cfu/ml
Milk shops	4.79	5.34	4.23	5.11	5.34
Milk Men	5.00	5.11	4.58	4.65	4.63
Dairy Farm	3.46	4.74	3.26	4.26	4.26
Collection center	4.18	4.81	3.54	4.18	4.99
Milk dairies	4.18	5.30	4.30	4.54	4.54

3.3.Prevalence of bacterial pathogens

Several bacterial pathogens were inaccessible and acknowledged from milk models. *Escherichia coli* were the most isolated organism which was found in 48 % (144/300) of the samples. *Staphylococcus aureus* was detected in 22% (66/300) of samples. *Salmonella spp* was present in 15% (45/300), while *Shigella* spp was present in 10% (30/300) ($P < 0.05$). E. coli was particularly present across all zones with the highest prevalence in Zone 2 (56 %), while *S. aureus* was most common in Zone 5 as shown in figure 1.

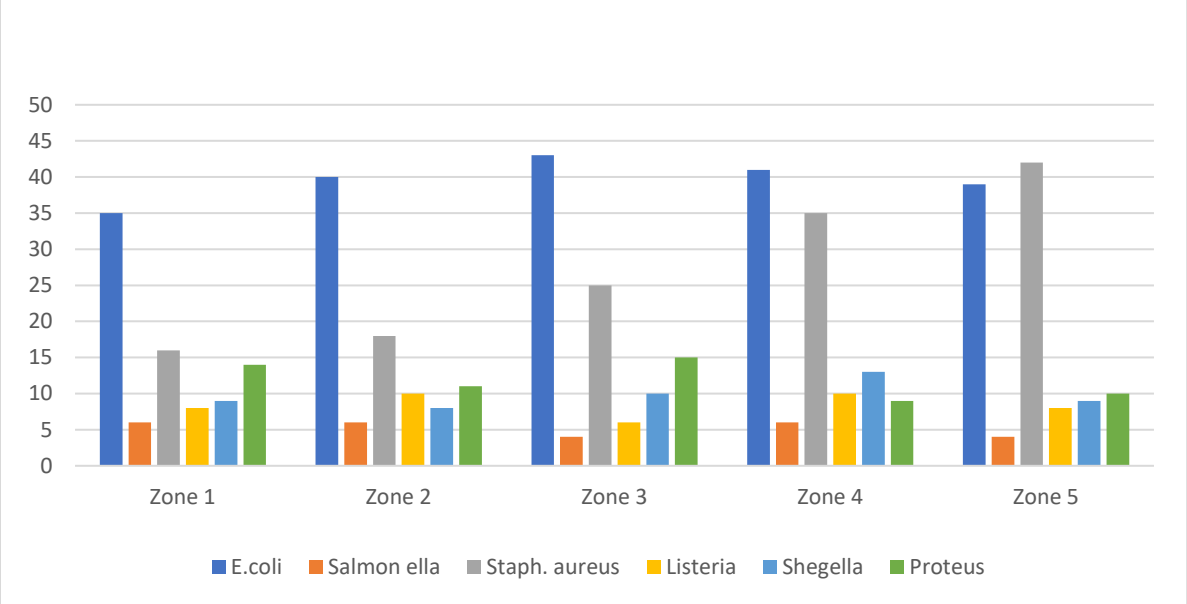

Fig 1: Rate of isolation of different bacteria from different zones of Multan

3.4.Methylene Blue Reduction Test (MBRT)

MBRT remained performed as rapid pointer of milk quality. Results showed that only 10% of milk samples retained their blue coloration for more than 5 hours were classified as very good quality. Approximately 20% of samples showed decolonization within 3-4 hours and were classified as good while 70% of samples showed decolonization within 1-2 hours which include them in fair to poor quality milk.

3.5.Antimicrobial resistance profiles

Antimicrobial exposure challenging was executed on insulates of *E. coli, Salmonella, S. aureus, Shigella, Listeria* and *Proteus spp* by via Kirby-Bauer disc dissemination technique. High levels of resistance were recorded in contradiction of frequently used antibiotics as ampicillin then doxycycline. Among *E. coli* isolates, 65% were resistant to ampicillin, 58% to doxycycline and 42% to streptomycin. However, lower resistance rates were observed for ciprofloxacin (12%), gentamicin (15%) and enrofloxacin (18 %). Similarly, *S. aureus* isolates show resistance to

ampicillin (60%) and doxycycline (54%), while showing higher susceptibility to ciprofloxacin (85%) besides gentamicin (80%).

On additional indicator, *Salmonella* isolates exhibited 48% fighting to ampicillin besides 45% to doxycycline, but only 10% resistance to enrofloxacin and 15% to gentamicin. *Shigella* isolates also followed better susceptibility to fluoroquinolones remains comparatively effective.

3.6.Multidrug resistance (MDR)

Resistant to three or additional courses of antibiotics was experiential between bacterial isolates. Midst *E. coli* insulates 38% were classified as MDR, 25% of *S. aureus* isolates and 18% of *Salmonella spp.* isolates. 12% of *Shigella* isolates were detected as MDR.

4. Discussion

The present study find contagious value besides antimicrobial resistance of uncooked milk marketed in Multan city, Pakistan. Findings show that majority of milk samples were contaminated with pathogenic bacteria. One important observation was the acidic pH values in many samples. Acidic pH in milk suggests microbial growth and presence of substances such as acids, hydrogen peroxide or preservatives. Recent studies also report similar results that milk samples had pH values below the optimal range which indicate spoilage (15).

Microbiological analysis revealed that *E. coli* and *S. aureus* were most common pathogens. These bacteria are well-known indicators of fecal, handlers or equipment contamination. Similar findings are reported in Bangladesh, where *E.coli* isolates from raw milk shows high prevalence and multidrug resistance (20). Similarly in Ethiopia, *S. aureus* was detected in ~25% of milk and milk product samples (17).

The detection of pathogens such as *Salmonella, Shigella, Proteus* and *Listeria* in raw milk is also concerning because of their potential for causing foodborne illness. Although their prevalence was lower compared to *E.coli* and *S. aureus* but can pose serious public health risks, especially

in populations consuming raw and unpasteurized milk. A study in Italy also confirmed that *E. coli*, *S. aureus* and *Listeria monocytogenes* are common in raw milk with contamination associated with inadequate hygiene practices (6).

Methylene Blue Reduction Test (MBRT) consequences in this study exposed maximum tasters decolored speedily which demonstrates high level of infectious motion. This line up through conclusions from Faisalabad, Pakistan, where underprivileged MERT notches interrelated with tall contagious heaps and meagre cleanliness (15). Antimicrobial resistance (AMR) forms pragmatic was regarding. High struggle to generally used antibiotics as ampicillin and doxycycline was renowned. Though, medicines as ciprofloxacin and gentamicin presented moderately improved effectiveness. Comparable shapes are described in Bangladesh, where high hostility was understood in contradiction of ampicillin. But, confrontation to fluoroquinolones was subordinate (20).

Detection of MDR isolates amid *E. coli* and *S. aureus* is troublesome. Bangladesh study originates that all *E. coli* insulates were MDR (20). Such conclusions reflect our alleged and accentuate that uncooked milk may act as tank for antibiotics impervious microbes and genetic factor, which could be conceded to individuals over straight ingesting or via corruption of dairy foodstuffs. Extensive attendance of morbific bacteria, particularly in milk located from sellers, procedures and assortment facts. In current study, dirty udders, contaminated water, unclean containers and poor handling were frequently observed. This is consistent with research in Faisalabad, which reported that improper milking techniques and handling correlated strongly with total bacterial count and coliform levels (15).

From a public health perspective, the consumption of raw or minimally processed milk contaminated with pathogenic and antibiotic resistant bacteria poses risks, especially among vulnerable population such as children, elderly or immune-compromised individuals. The risk is eventually high where pasteurization is uncommon or not strictly enforced. The review of raw milk and dairy products in Ethiopia shows how AMR in milk-borne pathogens is an emerging concern in many low and middle income countries (6).

5. Conclusion

This study demonstrated that raw milk sold in Multan city is contaminated with several pathogenic and opportunistic bacteria which include *E. coli, S. aureus, Salmonella, Shigella, Proteus* and *Listeria*. Presence of these microbes and acidic pH reflects underprivileged sanitariness throughout milking, packing and transference. Furthermore, recognition of MDR insulates specifies mounting antimicrobial fighting allied to overdoing of antibiotics in cattle and hominoid medication. Discoveries illustrate imperative requirement for enhanced disinfected performs then appropriate decontamination besides answerable drugs use to guarantee milk security and guard community wellbeing. Unvarying microbial scrutiny and cognizance mid milk creators and patrons are indispensable to avoid milk borne contaminations.

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