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Original Article

Detection of Coliform Bacteria in Raw Milk Samples Collected from Industrial Cities of Pakistan

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ABSTRACT

Antibiotics and heavy metals-resistant bacteria in livestock environments can result in economic losses and raise public health and environmental problems. There is a crisis in the world's access to and pipeline for antibiotics. **Objective:** To screen raw milk samples collected from three different industrial cities Gujranwala, Lahore, and Sheikhupura, situated in the province of Punjab, Pakistan. **Methods:** In this regard, a total of 26 samples were having 84 coliform strains. Separated coliform colonies were processed for Gram's staining, catalase, indole production, and Simmon's citrate and motility tests. **Results:** Metal resistance of bacterial strains was also checked and 39.5% and 45.23% of bacteria were found to be resistant to ZnCl2 1% and 0.5%. 69.045% and 77.38% bacteria were found to be resistant to CuSo4 salt solution1% and 0.5%. 17.85% and 27% bacteria were found to be resistant to Na2CrO4 salt solution1% and 0.5% respectively. 80% of bacteria were found to be resistant to Cefuroxime, 26.19% to Cephradine, 84.52% to Aztroeonam 41.67% to Erythromycin, 91.667% to Trimethoprim 89.28% to Lincomycins. **Conclusions:** The raw milk samples were not only contaminated with coliforms but the bacteria were also resistant to heavy metals and certain antibiotics which might be considered indicative of industrial and anthropogenic pollution. Cephradine, 84.52% to Aztroeonam 41.67% to Erythromycin, 91.667% to Trimethoprim 89.28% to Lincomycins. Conclusions: The raw milk samples were not only contaminated with coliforms but the bacteria were also resistant to heavy metals and certain antibiotics which might be considered indicative of industrial and anthropogenic pollution.

INTRODUCTION

Since the start of 20th-century, agriculture mechanization, industrialization, and urbanization have been causing an increase in metals and antibiotics resistance among the bacteria. Major milk-producing countries are the United States, Russia, India, and Pakistan is ranked as fourth. Pakistan has 45 million tons of annual milk production. The rural area population's (30-40%) income source is solely the rearing of livestock. In Pakistan, there are 34 million cattle and 31 million buffaloes. Buffaloes contribute to 62% of milk and are major milk producers [1]. Black gold buffaloes in Pakistan are a major milk source as their milk is consumed by all age groups [2]. Coliforms are also lactose fermenting bacteria including several genera like Escherichia, Citrobacter, Enterobacter, and Klebsiella. They are considered the indicator of fecal contamination. They normally live in the intestines of humans and animals. Their presence in milk is of severe health concern. In Pakistan, milking practices are very poor and unhygienic [3]. To increase the reproductive potential heavy metals are extensively used as a therapeutic agent in farms [4]. Heavy metals represent major contaminants with severe health and environmental problems [5, 6]. Due to their presence in nature and persistence, heavy metals are marked as hazardous to ecosystems and human beings

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[7]. The presence of heavy metals in numerous media is of great concern. Irrigation of agricultural land with sewage and industrial wastewater is the basic cause of the development of heavy metal resistance [8]. Food items grown in contaminated soil or with wastewater are reservoirs of heavy metals and transfer agents of heavy metals to humans and animal bodies [9]. Animals consuming contaminated fodder produce milk contaminated with heavy metals [10]. Heavy metal accumulations in dairy animals result in their excretion in milk [11]. Milk consumption of cattle and buffalo which had been feeding at polluted places leads to several human health problems [12, 13]. The livestock industry uses antibiotics and heavy metals as the most common supplements [14]. Antibiotic resistance has become one of the biggest issues in the world in treating bacterial infections [15]. During livestock production, use of antimicrobial agents causes the development of antimicrobial resistance which is a serious health concern for the public. By increasing the antibiotics in the environment, antibiotic resistance is more pronounced in the environment [16, 17]. Usage of drugs in animal food is a vital tool for animal welfare and health. Despite various health benefits of antimicrobial drug usage, the production of antimicrobial-resistant bacteria is also an adverse condition [18]. There is a positive correlation between increases in antibiotic resistance among disease-causing bacteria with the application of antibiotics to farming animals [19]. Further evolution and increase in antibiotic resistance in pathogenic microbes enhance the severe health issues for animals and humans [20]. When food containing the antimicrobials is given to animals outside the regimen dose label and durations then antimicrobial resistant microbes are produced [21]. The use of antibiotics in growth promoters and medicine causes an increase in antibiotic-resistant bacteria [22]. Bacteria showing antibiotic resistance have been found in milk, vegetables, cheese, meat, and fruits [23]. Coliforms having antibiotic resistance are pathogenic bacteria that cause water pollution and environmental and public health issues in developing countries [24, 25]. Genes for antibiotic resistance are localized in mobile genetic elements and are transferred by bacteria in the food to the bacteria living in the human body by a process of horizontal gene transfer. Strains of E. coli are naturally more abundant in the gastrointestinal tract [26)]. From the previous 50 years, E. coli has increased in multidrug resistance by 56.4%. This spread might be due to bacteria belonging to different ecosystems because resistance genes are localized on mobile vectors [26].

The present study focused on the raw milk quality in terms of antibiotics and heavy metals resistance bacterial content of three cities.

METHODS

Sample Collection

Twenty-six samples were collected, from three cities Gujranwala, Lahore, and Sheikhupura of Punjab Province, Pakistan. Raw milk samples were collected in autoclaved sterile bottles from local shops and livestock farms, from 6 November 2017 to 25 May 2018. they were transported to Microbiology Lab, Zoology Department, University of Punjab, Lahore, Pakistan, and stored at 40C till further use.

Processing of Samples for Bacterial Colony-Forming Units

In 99 ml of distilled autoclaved water, 1 ml of raw milk was mixed. From this dilution 1ml was, mixed with 99 ml of distilled water and so on [27]. From diluted milk 0.1 ml was spread on EMB agar with the help of a spreader and incubated for 24 hours at 370C (27). Colony-Forming Unit (CFU) has then been enumerated for each category of the bacterial colonies. One separated colony was picked up with the help of a sterile loop and streaked on Nutrient agar, using the quadrate streaking method. The plates were incubated routinely and a well-separated colony from nutrient agar was picked up and again streaked on EMB agar to get pure culture. After getting pure culture characterization of bacterial colonies i.e., configurations, margins, elevation, color, and size were determined and noted [28]. A total of 78 bacterial isolates were processed.

Biochemical Analysis

Differential Grams staining test, motility test, catalase test, indole test, and Simmons citrate agar production test were performed [29], using pure cultured bacteria. In bacterial strain identification, "S" represents Shiekhupura; "L" represents Lahore and "G" represents Gujranwala as well as in milk sample labeling.

Metal Sensitivity Test

To check metal susceptibility Cu, Cr, and Zn salts solutions like (CuSo4), (Na2CrO4) and (ZnCl2) with 1% and 0.5% concentrations were used, and discs from filter paper of equal size were cut, inoculated with 6 µl of each salt solution and these disc were autoclaved for 20 minutes at 1210C temperature and 15 psi pressure, these inoculated autoclaved disc were placed on nutrient agar plates aseptically and incubated for 24 hours at 370C temperature and metal sensitivity results were recorded [30].

Antibiotic Susceptibility Test

Commercially available antibiotics which include Cefuroxime (30mcg), Flumequine (30mcg), Erythromycin (15mcg), Norfloxacin (10mcg), Tobramycin (10mcg), Cephradine (30mcg), Aztreonam (30mcg), Lincomycin (2mcg), Trimethoprim (5mcg) nine antibiotics were used to check cell wall, proteins and nucleic acid inhibition. They were placed on nutrient agar plates and incubated these antibiotics-containing plates for 24 hours at 37 0C temperature. This whole process is performed by using the

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Kirby-Bauer disc diffusion technique [31, 20].

RESULTS

In the table 1, first column represents the number of samples. In "G1a" G represents Gujranwala "1" represents the sample number and "A" represents strain type. Similarly, all other strains are represented in the same way, bacteria like *Salmonella* or *Shigella spp*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Escherichia coli*. All bacteria were found grams negative in staining

Table 1: Number of CFU per Milliliter of the Original Milk Samples and Presumptive Identifications of the Coliform Isolates based on their Phenotypic Characteristics

Sample No.	CFU/ml	Presumptive Isolates of Bacteria	No. of Strains	Isolate Code (Citrate; Motility; Indole & Catalase Production)
G1	1.48×10^4	*Klebsiella pneumoniae*	3	G1A (+,+ ,+&+); G1B (+,_ ,+ &_) ; G1C (+_+&+)
		Enterobacter cloacae	1	G1D (+,_,+&_)
G2	3.8×10^8	*Salmonella or Shigella spp.*	1	G2A (_,+,+&+)
		Klebsiella pneumoniae	1	G2B (+,+, + &+)
		Enterobacter cloacae	2	G2C (_, _ ,+ &+); G2D (+,_, _&+)
G3	6.88×10^4	*Enterobacter cloacae*	2	G3A (+,_,+&_); G3D (+,_,+&+)
		Salmonella or Shigella spp.	1	G3B (_,_,+&+)
		Klebsiella pneumoniae	1	G3C (_,+,+&_)
G4	1.56×10^8	*Klebsiella pneumoniae*	1	G4A (_, _,+&_)
		Enterobacter cloacae	4	G4B (_, _ ,+ &+); G4C (_,+,+&+); G4D (_,+, +&_); G4F (+,_,+&+)
		Salmonella or Shigella spp.	1	G4E (+,+, +&+)
G5	5.4×10^8	*Enterobacter cloacae*	1	G5A (_,_,+&+)
G6	1.8×10^6	*Enterobacter cloacae*	2	G6A (_, _ ,+&_); G6C (+,_,+&_)
		Klebsiella pneumoniae	1	G6B (+,_,+&+)
G7	2.8×10^6	*cloacae*	2	G7A (_,+,+ &+); G7B (+,_,+&+)
		Klebsiella pneumoniae	1	G7C (+,+, +&+)
G8	1.52×10^4	*Klebsiella pneumoniae*	2	G8A (+,_,+&_); G8C (_,+, +&+)
		Enterobacter cloacae	1	G8B (+,_,+&_)
G9	4.8×10^5	*Enterobacter cloacae*	2	G9A (+,_,+&_); G9B (_, _ ,+&+)
L1	5.6×10^4	*Klebsiella pneumoniae*	2	L1A (+,_,+&+); L1B (_, _,+ &+)
		Enterobacter cloacae	1	L1D (_,+,+& +)
L2	6.0×10^4	*Klebsiella pneumoniae*	2	L2A (_,_,+ &+); L2B (+, + ,+&+)
		Enterobacter cloacae	3	L2C (+,+,+&+)
L3	3.5×10^6	*Klebsiella pneumoniae*	2	L3A (+,+, _&+); L3B (+,_, _&+);L3C (+,+,+&_)
L4	2.99×10^{10}	*Klebsiella pneumoniae*	2	L4A (_,+, +&+); L4B (_, _ ,+&+)
L5	3.04×10^6	*Klebsiella pneumoniae*	2	L5A (+,_,+&_); L5C (+,_,+&+)
		Enterobacter cloacae	1	L5B (_, _ ,+&_)
S1	7.2×10^6	*Escherichia coli*	1	S1A (+,_,+ &+)
		Klebsiella pneumoniae	1	S1B (+,_,+ &+)
		Enterobacter cloacae	1	S1D (_,+,+&_)
S2	6.0×10^6	*Enterobacter cloacae*	2	S2A (_,_,+&_); S2C (_, _ ,+ &+)
		Klebsiella pneumoniae	1	S2B (_, _ ,+& +)
S3	3.1×10^7	*Enterobacter cloacae*	2	S3A (_,_,+ &_); S3B (_,+,+ &+)
S4	4.6×10^6	*Klebsiella pneumoniae*	2	S4A (_, _ ,+ &+); S4C (_,+,_& _)
		Enterobacter cloacae	1	S4B (+,+,+ &+)
S5	3×10^7	*Klebsiella pneumoniae*	2	S5A (_,_,+ &+); S5B (_,+, +&+)
S6	7.4×10^8	*Klebsiella pneumoniae*	1	S6A (+,_,+&+)
S7	5.69×10^8	*Enterobacter cloacae*	1	S7A (_,+,+&+)
		Klebsiella pneumoniae	2	S7B (_,+, +&+); S7C (+,_,+&+)
S8	3.1×10^8	*Enterobacter cloacae*	2	S8A (+,+,+&+); S8C (+,_,+&_)
		Klebsiella pneumoniae	2	S8B (_,+,+&_); S8D(_ ,_ ,+ &+)
S9	3.4×10^8	*Escherichia coli*	1	S9A (_, _ ,+&_)
		Klebsiella pneumoniae	2	S9B (_,+, _ &_); S9C (+,_,+& +)

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S10	6.23×10^8	*Enterobacter cloacae*	2	S10A (_,+, + &+); S10C (+,+, +&_)
		Klebsiella pneumoniae	1	S10B (_, _ ,+&+)
S11	7.2×10^8	*Klebsiella pneumoniae*	2	S11A (+,+,+&+); S11C (+,_,+&+)
		Enterobacter cloacae	1	S11B (_,+, +&+)
S12	8.0×10^8	*Escherichia coli*	2	S12A (_,+,+&_); S12C (+,_,+&_)
		Klebsiella pneumoniae	2	S12B (_,+,+&_); S12D(_,+, _ &_)

Figure 1 shows the prevalence of different species identified in different cities.

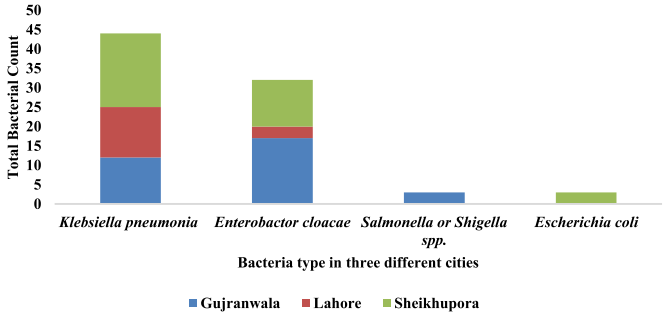

Figure 1: Presumptive Identifications of the Bacterial Isolates from Milk Samples of Gujranwala, Lahore and Sheikhupura.

Viable Bacterial Count

The CFU data corresponding to the cities of Sheikhupura, Lahore, and Gujranwala were statistically analyzed using the Kruskal-Wallis test a non-parametric, alternative to one ANOVA. Results showed significance at $p<0.05$ (table 2). The bacterial count was high in Sheikhupura which was collected from local dairy shops. Lahore and Gujranwala showed significantly lower viable bacterial counts as they were collected directly from cow udder and milkmen's buckets, respectively. Milk samples of Lahore city were taken directly from animals. In the case of Sheikhupura city milk samples S1-S12 were taken from local shops. Samples G1 to G5 all were taken from milkman buckets, whereas samples G6 to G9 and L1-L5 were taken directly from animals.

Table 2: Results of Kruskal-Wallis Test of milk samples collected from different cities

City	Mean Rank CFU/mL	Test Units (No. of samples)	p-value
Gujranwala	9.33	9	0.024
Lahore	10.40	5	
Sheikhupura	17.93	12	

Following the significance Post-Hoc test applied, test results are shown in figure 2 which shows that milk samples from Gujranwala and Sheikhupura were significantly different from each other while the comparison of Sheikhupura – Lahore and Gujranwala-Lahore showed to be non-significant-respectively at $p< 0.05$(figure 2).

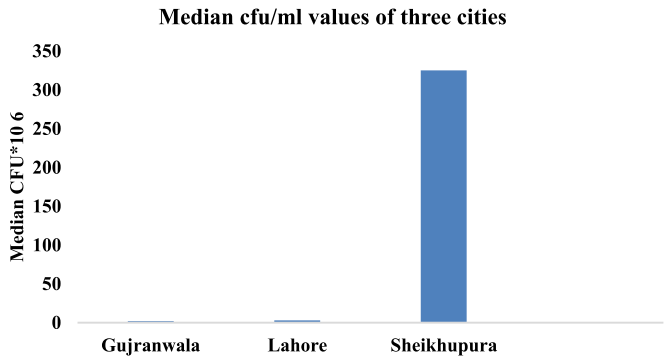

Figure 2: CFU\ml Median values of milk samples collected from Gujranwala, Lahore and Sheikhupura, letter showing significance at $p<0.05$.

Metals and Antibiotics Resistance of Coliforms

When bacterial isolates were grown in the presence of 1% and 0.5% (CuSo4), (Na2CrO4), and (ZnCl2) salts of zinc (Zn), Copper (Cu), and Chromium (Cr). 39.5% and 45.23% bacteria were found to be resistant to ZnCl2 salt solution1% and 0.5% concentrations. 69.045% and 77.38% bacteria were found to be resistant to CuSo4 salt solution1% and 0.5% concentrations. 17.85% and 27% bacteria were found to be resistant to Na2CrO4 salt solution1% and 0.5% concentrations respectively. Antibiotic susceptibility of coliform isolates was also tested against nine different antibiotics with three modes of action i.e.- cell wall inhibitors, proteins inhibitors, and nucleic acid synthesis inhibitors.79.76% bacteria were found to resistant for Cefuroxime, 26.19% bacteria were found to resistant for Cephradine, 84.52% bacteria were found to resistant for Aztroeonam 41.67% bacteria were found to resistant for Erythromycin,91.667% bacteria were found to resistant for Trimethoprim 89.28% bacteria were found to resistant for Lincomycins as shown in figure 3(a) and 3(b).

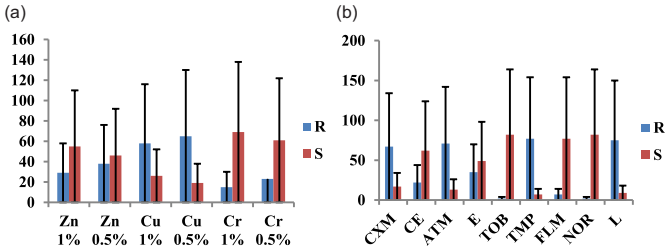

Figure 3: Mean sensitivity and resistance of coliforms against different metals (a) and antibiotics (b). S=Sensitive R=Resistant; results are the mean of three replicates.

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DISCUSSION

The milk of a healthy cow is free from any contamination when secreted into the alveoli of the udder. Fresh milk contains a very low microbial load ranging few to 1000 CFU/ml [32, 33]. Microbial contamination might result during milking storage, handling, refrigeration, and transportation increasing microbes by 100 or even 1000 folds. In the present research, the total bacteria count taken directly from the udder during milking (L1, L2, G8) was relatively low ranging at 10^4 CFU/ml. An increase in bacterial CFU/ml was observed from milk during its transportation from the milk farm to milk containers as (G1 to G5) samples were taken from buckets. It showed a bacterial average of 2.15×10^8 CFU/ml which is manyfold high as compared to milk taken directly from the udder which is approximately in according to the findings Sandholm *et al.*, who observed 10^3 to 10^{11} CFU/ml due to post-harvest milk treatment [34]. All milk samples of Lahore city taken from directly under udder represented in as (L3, L4, L5) had a mean bacterial count of 9.97×10^9 CFU /ml which is very high and contradicting the findings of Mutukumira *et al.*, who found the coliform bacteria 3.2×10^2 to 2.3×10^5 range [35]. Samples of Gujranwala coded as (G6, G7, G9) which were collected from farms also disagreed with the result of Khan *et al.*, who recorded total coliform count < CFU1000/ml [36], but correlated to the findings of Uddin *et al* (37). Samples taken from Sheikhupura represented as (S1-S12) were taken from local shops and showed a relatively very high mean count which is 3.48×10^8 CFU/ml which is compatible with the previous studies [38, 39]. The majority of milk samples did not meet the UE no. 1662/2006 law regulation committee which reported that cow's milk samples should not increase 1×10^5 CFU mL^{-1} [40]. In all milk samples, average milk bacterial count was high as compared to the national average raw milk bacterial count [41]. This variation shows different storage temperatures and time, seasonal variation, and higher bacterial count during summer time [42]. Different CFU/ml count at farms predicts different hygienic milk handling practices and different environmental and seasonal variations. High bacterial load predicts traditional milking practices, high milk fecal contamination, and milk adulterations at farms [43]. During the transportation chain, various factors like farm milk adulteration, transportation of milk in the absence of controlled temperature and its transportation in poorly cleaned bottles and tanks have enhanced milk contaminations [44]. High coliform load in milk shows fecal as well as environmental contaminations resulting from poor hygienic practices during milking, handling, unhygienic water use, and transportation [45, 46]. Contaminated milk not only shows public health issues but also represents poor milk quality [47]. Heavy metal accumulations in dairy animals result in their excretion from milk [11]. Consumption of such contaminated milk by people results in serious health concerns [13]. 69.045% and 77.38% bacteria were found to be resistant to $CuSO_4$ salt solution at 1% and 0.5% concentrations. It is considered that *Enterobacteriaceae* of cow raw milk samples are the fountain of antibiotic resistance genes [48, 23]. Antimicrobial resistance is considered as a form of pollution [49]. Commercially available antibiotics which include Cefuroxime (30mcg), Flumequine (30mcg), Erythromycin (15mcg), Norfloxacin (10mcg), Tobaramycin (10mcg), Cephradine (30mcg), Aztreonam (30 mcg), Lincomycin (2mcg), trimethoprim (5 mcg) nine antibiotics used to check cell wall, proteins and nucleic acid inhibition. Most 79.76% of bacterial strains were resistant to CXM, 84.52% of bacterial strains were resistant to ATM, 91.667% of bacterial strains were resistant to TMP 89.28% of bacterial strains were resistant to L these results correlated with Araque *et al.*, Murdoch *et al.*, and Bagré *et al.*, whose test results showed most coliforms were resistant to different antibiotics [50-52]. Other coliform strains did not show such results. Most bacterial strains were sensitive to NOR and TOB. Antibiotic resistance of bacterial isolates of milk is agreed with literature findings [53, 54]. Coliforms are gram's negative rods agreed with the [55]. Coliform mostly were non-motile and least motile. Some coliform strain has catalase enzymes that were in support of scientific findings [56, 57]. Bacteria having catalase enzyme produced bubbles on adding a few drops of H_2O_2. Tryptophanase enzyme is present in most coliforms and present research indole positive test results are in favor of previously reported results [29, 58, 59]. *Escherichia coli* can be identified with Eosin Methylene Blue (EMB) agar. The presence of green-metallic sheen in three strains of E. coli was observed in milk sampled from Sheikhupura whose results agreed with previous findings [60, 61]. The citrate utilization test on Simmons citrate agar and several strains gave citrate positive test by changing the green color of media into blue these results are compatible with previous research [54].

CONCLUSIONS

Coliforms are indicators of fecal contamination. Their presence in large numbers predicts the very poor hygienic condition of the study area, even milk taken directly from the farm was not acceptable for consumption. So, dairy farm owners should be educated to render fresh milk suitable for human consumption. Many coliforms were resistant to four antibiotics and copper showing environmental pollution and its influence on our diet. As coliforms are mostly fecal in origin, their presence in milk is a health hazard emphasizing to improve milk handling.

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Authors Contribution

Conceptualization: JIQ
Methodology: AA, MAR
Formal analysis: AA, AH
Writing-review and editing: AA

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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