COMPARATIVE STUDY TO DETECT ANTIBIOTIC RESIDUES IN PROCESSED AND RAW MILK IN LAHORE

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ABSTRACT: Antibiotics help animals and human beings live longer and healthier lives. But if antibiotics are used carelessly or inappropriately, or if the required withdrawal period is not understood, it's possible that treated animals' products will include unintended antibiotic residues. Many of these life-saving drugs are losing their efficacy as formerly vulnerable microorganisms (bacteria, viruses, fungi, and microscopic parasites) develop resistance. This phenomenon is called "antimicrobial resistance" or AMR. Antimicrobial usage (AMU) is a significant risk factor for antimicrobial resistance (AMR) development. This study aimed to investigate the presence of β-lactam and tetracyclines antibiotic residues in raw (cow and buffalo) milk and processed (UHT and pasteurized) milk in District Lahore. For that purpose, a total of 200 milk samples, containing 100 raw milk samples and 100 processed milk samples were collected from 5 zones of Lahore. The samples were processed using Milk Antibiotic Residues Rapid Test (Bioeasy) for β-lactams and tetracyclines in the milk kit method which is based upon colloidal gold immunochromatography technology also known as lateral flow dipstick immunoassay. Among the total of 200 milk samples, the results showed that 6.5% of milk samples were positive for only beta-lactams, 1.5% of milk samples were positive for only tetracyclines, 9.5% of milk samples exhibited dual positivity for both beta-lactams and tetracyclines, 8.5% milk samples demonstrated weak positive results for both antibiotics, and 74% milk samples were negative for beta-lactams and tetracyclines both. The calculated p-value (0.0004998) indicates a significant difference between the observed samples in terms of antibiotic residue presence by Fisher's exact test. These residues were more in the raw milk as compared to the processed milk. Regulatory authorities should ensure a proper withdrawal period before milking the animals and definite supervision is necessary on the application of these drugs as this is a serious healthcare concern in Lahore.

Key words: Antibiotics, AMR, AMU, Milk, Antibiotic residues, β-lactams, tetracyclines

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INTRODUCTION

Milk is a rich source of almost all the essential elements required for the development and well-being of human health. Antibiotics are vital medications for treating a variety of illnesses in both animals and human beings (Garcia *et al*. 2020). The most popular antibiotic classes in veterinary practices may include β-lactams and tetracyclines for the treatment of different illnesses and their prevention, growth stimulation, and boosting the production efficiency of animals and their products. However, inappropriate and imprudent antibiotic use, as well as a lack of understanding of the compulsory withdrawal period might result in unwanted antibiotic residues appearing in the milk of treated animals (Kumar *et al*. 2021). Antimicrobial usage (AMU) is a significant risk factor for antimicrobial resistance (AMR) development (Mikecz O *et al*. 2020). One of the major global health challenges to people today is antibiotic resistance (Zhou *et al*. 2018). ß-lactam residues at maximum residues limit (MRL) or even below the limit may cause the coagulation of sheep milk yogurt to take 40 minutes longer, which will affect the quality of the final product. Humans get allergic responses and other chronic health issues after consuming antibiotic residues in milk (Kyuchukova R. 2020). Moreover, the consumption of even tiny dosages of antibiotics might enable resistant bacteria to proliferate selectively in the intestines, leading to their overgrowth (Khanal *et al*. 2018).

Different diseases like foot and mouth disease, blue tongue, hemorrhagic septicemia, anthrax, brucellosis, mastitis, listeriosis, leptospirosis, lumpy skin disease, and worm infestation harm the livestock in

Pakistan and cause significant economic losses. Antibiotics along with other drugs are used to treat these diseases Van den Bogaard AE and Stobberingh EE (1999). Besides the recovery of animals, these drugs also have some harmful consequences on animal and human health as they consume these products (Ahmed *et al*. 2020).

The oldest group of antibiotics, known as ß-lactam, is frequently used in Pakistan to treat ill animals. Lack of use of proper farming practices increases dairy milk contamination with antibiotic residues (Berruga MI *et al*. 2016). Milk of these animals is consumed without any precautions which results in much harm to the dairy industry as well as to human health. Maximum residual limits (MRL) for each antimicrobial agent have been established by legislation in several countries to reduce the risks associated with antibiotic residues (Comunian R *et al*. 2010).

Antibiotics are administered indiscriminately and proper withdrawal periods are rarely observed in the country as a result of a lack of awareness and practical dairy principles. These antibiotic residues might be harmful to both human and animal health, and they would be a major obstacle to the export of milk (Chowdhury S *et al*. 2015). Governments all around the world have set up monitoring systems to identify the amounts of antibiotic residues in food and establish a maximum residual level (MRL) for them. To find antibiotic residues in milk, regulatory bodies utilize several analytical methods, including high-performance liquid chromatography (HPLC), gas chromatography combined with mass spectrometry (CG-MS), and antimicrobial detection kits. The current study was conducted for the determination of antibiotic residues in processed and raw milk which is a serious healthcare concern in Lahore.

MATERIALS & METHODS

The current study was organized with the idea of the detrimental effects of antibiotic usage in animals on both human and animal health and to access the investigation of commonly used antibiotics (beta-lactam and tetracycline) residues in processed and raw milk in 5 zones of district Lahore.

Sampling Strategy: A total of 200 milk samples containing 100 milk samples of buffalo and cow were collected from open markets, local vendors, middlemen, and various farms as raw milk. 100 samples of UHT and pasteurized milk of different brands were collected from local markets as processed milk. The number of samples collected from different zones is as under:

Table 1. : Number of samples.

S. No.	Zones of Lahore	No of samples (raw milk of cow)	No samples (raw milk of buffalo)	No of samples (Processed UHT)	No of samples (processed pasteurized milk)
1.	North and vicinity	10	10	10	10
2.	South and vicinity	10	10	10	10
3.	West and vicinity	10	10	10	10
4.	East and vicinity	10	10	10	10
5.	Center and vicinity	10	10	10	10

Milk samples were procured in falcon tubes. Appropriate ice boxes were used for transportation and then these samples were kept in a refrigerator at 4°C till processing.

Sample processing and testing method: To detect antibiotic residues, the Milk Antibiotic Residues Rapid Test (Bioeasy) for β-lactams and tetracyclines in the milk kit method (kit ID: YRM1007-401) was used. This kit is utilized to detect β-lactams and tetracyclines in milk. This kit is based on the colloidal gold immune-chromatography technique.

Test preparation: First of all the incubator was turned on and waited till the temperature stabilized at 40°C ± 2°C. Then the kit was collected from the refrigerator and the test tubes were allowed to warm up to room temperature (15-30°C ± 2°C). After warming up the test tubes the required number of microwells and dipsticks from the test tube were taken (see Figure 1). Milk samples were mixed well so that they become homogenous before testing.

Test procedure: Milk samples were pipetted 200μl (see figure 2) into the reagent microwell and were mixed well by pipetting up and down 5-10 times (see figure 3). Then it was incubated for 3 mins at 40°C ± 2°C. After incubation, the dipstick was inserted into the microwave. Then it was again incubated for another 3 mins at 40°C ± 2°C. After the second incubation, the dipstick from the microwells was taken out and the sample pad was removed at the lower end. The results were interpreted according to the interpretation table provided with the Kit brochure.

Figure 1: Microwells and dipsticks Figure 2: Taking 200µl milk sample Figure 3: Pouring 200µl milk sample in microwell before inserting the dipstick.

RESULTS

A total of 200 samples were collected from open markets, local vendors, middlemen, and various farms as raw milk. UHT and pasteurized milk of different brands were collected from local markets as processed milk. 100 samples of raw milk (20 samples from each zone) were collected. The 20 samples of each zone were further divided into two categories, Category A: 10 samples of cow milk were collected, and Category B: 10 samples of buffalo milk were collected. 100 samples of pasteurized milk (20 samples from each zone) were collected. The 20 samples of each zone were further divided into two categories, Category A: 10 samples of UHT milk were collected, and Category B: 10 samples of pasteurized milk were collected. The samples were undergone through bioeasy milk antibiotic rapid test for β-lactams and tetracyclines in milk kit method (Kit ID: YRM1007-401). The results per zone wise delineated were as follows:

Table 2: Overall summary of milk samples results tested for antibiotic residues. The observed data has been presented as n (% out of the total sample in the table) along with the 95% Confidence interval [95% C.I].

Category	β-lactams positive	Tetracyclines positive	Both positive	Weak positive	Both negative	*P-value*
Raw milk of cow	8 (4%) [7.69-2.04]	0 (0%) [1.88-0]	8 (4%) [7.69-2.04]	8 (4%) [7.69-2.04]	26 (13%) [18.37-9.03]	*0.0005*
Raw milk of buffalo	4 (2%) [5.03-0.78]	3 (1.5%) [4.32-0.41]	3 (1.5%) [4.32-0.41]	6 (3%) [6.39-1.38]	34 (17%) [22.82-12.43]	
Processed UHT milk	1 (0.5%) [2.78-0.03]	0 (0%) [1.88-0]	2 (1%) [3.57-0.18]	3 (1.5%) [4.32-0.41]	44 (22%) [28.24-16.82]	
Processed pasteurized milk	0 (0%) [1.88-0]	0 (0%) [1.88-0]	6 (3%) [6.39-1.38]	0 (0%) [1.88-0]	44 (22%) [28.24-16.82]	

The study investigated the presence of two types of antibiotic residues, β-lactams, and tetracyclines, in different categories of milk samples, including raw milk of cow, raw milk of buffalo, processed UHT milk, and processed pasteurized milk. The data presented in Table 1 includes the number of positive samples and the percentage of positive samples out of the total observed samples in each category. Additionally, Fisher's exact test was employed to analyze the data, and the corresponding p-values were provided to assess the significance of differences between the observed samples in different categories. In the raw milk of cows, 8 samples (4%) tested positive for β-lactams, while none of the samples showed the presence of tetracyclines. Eight samples (4%) exhibited dual positivity for both β-lactams and tetracyclines. Similarly, 8 samples demonstrated weak positive results for both antibiotics. On the other hand, the majority of raw cow milk samples, totaling 26 (13% of observed samples), tested negative for both β-lactams and tetracyclines. The calculated p-value (0.0004998) for this category indicates a significant difference between the observed samples in terms of antibiotic residue presence. In the raw milk of buffalo, 4 samples (2%) tested positive for β-lactams, and 3 samples (1.5%) showed the presence of tetracyclines. Only 3 samples showed dual positivity for both antibiotics, and 6 samples showed only marginally positive results. In this category, 34 samples (17%) tested negative for both β-lactams and tetracyclines. In processed UHT milk, the presence of β-lactams was detected in 1 sample (0.5%), while no instances of tetracyclines were found. Two samples (1%) showed simultaneous positivity for both antibiotics, and 3 samples exhibited weak positive results. The majority of processed UHT milk samples, totaling 44 (22%), tested negative for both β-lactams and tetracyclines. Processed pasteurized milk demonstrated no instances of antibiotic positivity for either β-lactams or tetracyclines. However, 6 samples (3%) exhibited dual positivity for both antibiotics, none came weak positive, while the majority of the samples, totaling 44 (22%), were negative for both β-lactams and tetracyclines. Based on the p-values, it is evident that there are significant differences between the observed samples in the raw milk of cows. As the p-value of Fisher's exact test showed a significant difference in the number of observed samples among the different categories of milk samples of the whole sampling from all the study zones, a multiple comparison of the cells in each row and column was performed and to identify the significantly different category. Table 2 shows the matrix based on p-values of the cell-wise multiple comparisons across each row and column in the contingency table.

Table 3: Shows *p-value* based matrix of the multiple comparison of the number of observed samples row-wise and column-wise.

Category	β-lactams positive	Tetracyclines positive	Both positive	Weak positive	Both negative
Raw milk of cow	*0.004*	*0.574*	*0.09*	*0.039*	*0.0001*
Raw milk of buffalo	*0.740*	*0.014*	*0.41*	*0.37*	*0.26*
Processed UHT milk	*0.191*	*0.57*	*0.16*	*0.57*	*0.009*
Processed pasteurized milk	*0.041*	*0.574*	*0.57*	*0.007*	*0.009*

The values in the matrix represent p-values resulting from multiple comparisons to assess the significance of the number of samples identified in each cell of the contingency table, concerning the combination of milk sample category and antibiotic type. Upon analysis of the data, it was observed that for the β-lactams category, the raw milk of the cow demonstrated a significantly low p-value of 0.004, indicating a notable difference in the number of samples identified compared to the other milk categories for this antibiotic. On the other hand, the raw milk from buffalo had an astoundingly high p-value of 0.74, indicating that the total number of samples was not significantly different from those of the other milk categories. There was no noticeable difference in the number of samples discovered compared to the other milk types for tetracyclines since both the raw cow's milk and the UHT milk had equal p-values of 0.57. Processed pasteurized milk displayed a much lower p-value (0.01) when compared to the other milk categories, showing a wide variation in the number of samples discovered. The presence of both antibiotics in buffalo raw milk was also significantly different from other milk categories when both antibiotics were considered, with a p-value of 0.41. Furthermore, the UHT milk that had been treated had a moderately low p-value of 0.16, showing a significant difference in the number of samples found to contain both antibiotics. In contrast, the raw milk of cow and processed pasteurized milk exhibited notably higher p-values of 0.09 and 0.57, respectively, indicating no significant difference in the number of samples identified in relation to the presence of both antibiotics. Moreover, weak positive signals were observed for the raw milk of cow and processed pasteurized milk with p-values of 0.039 and 0.007, respectively. These weak positive signals suggest a minor variation in the number of samples identified compared to the other milk types in relation to both antibiotics. Lastly, all milk categories displayed a negligible p-value for the scenario where both antibiotics were absent, with values ranging from 0.0001 to 0.26. This indicates no significant difference in the number of samples identified when both antibiotics were not detected. The matrix is visualized in the form of a heat map generated by R software as follows.

Table 4 presents data regarding zone 1 and consists of information related to the presence of β-lactams and tetracyclines in different categories of milk samples, including raw milk of cow, raw milk of buffalo, processed UHT milk, and processed pasteurized milk. The data is represented in the form of n (the number of samples observed in each cell) and the percentage is calculated as the proportion of observed values out of the total observed samples in each category. The p-values obtained from Fisher's exact test are also provided to determine the significance of differences between the observed samples in different categories. In the raw milk of cows, 3 samples (7.5%) tested positive for β-lactams, while no instances of tetracyclines were found. Three samples (7.5%) demonstrated dual positivity for both β-lactams and tetracyclines. Additionally, 1 sample (2.5%)

exhibited a weakly positive response for both antibiotics and the majority of raw cow milk samples, totaling 3 (7.5% of observed samples), tested negative for both β-lactams and tetracyclines. The corresponding p-value for this category is 0.1039, indicating that the differences in antibiotic presence among the subgroups are not statistically significant. β-lactams were detected in one sample (2.5%) of buffalo raw milk, and tetracyclines were detected in one sample (2.5%). Two samples (5%) demonstrated simultaneous positivity for both antibiotics, while no weak positive results were observed. However, 6 samples (15%) were negative for both β-lactams and tetracyclines. In processed UHT milk, 1 sample (2.5%) showed β-lactams positivity, while no instances of tetracyclines were found. None of the samples exhibited dual positivity for both antibiotics and weak positive results were also absent. However, the majority of processed UHT milk samples, totaling 9 (22.5%), tested negative for both β-lactams and tetracyclines. Processed pasteurized milk demonstrated no instances of antibiotic positivity for either β-lactams or tetracyclines. However, 2 samples (5%) exhibited dual positivity for both antibiotics, while the majority of the samples, totaling 8 (20%), were negative for both β-lactams and tetracyclines. Regarding the significance of differences, based on the p-values provided, the analysis indicates that there is no statistically significant difference in antibiotic presence between the subgroups (*p*-value > *0.05*).

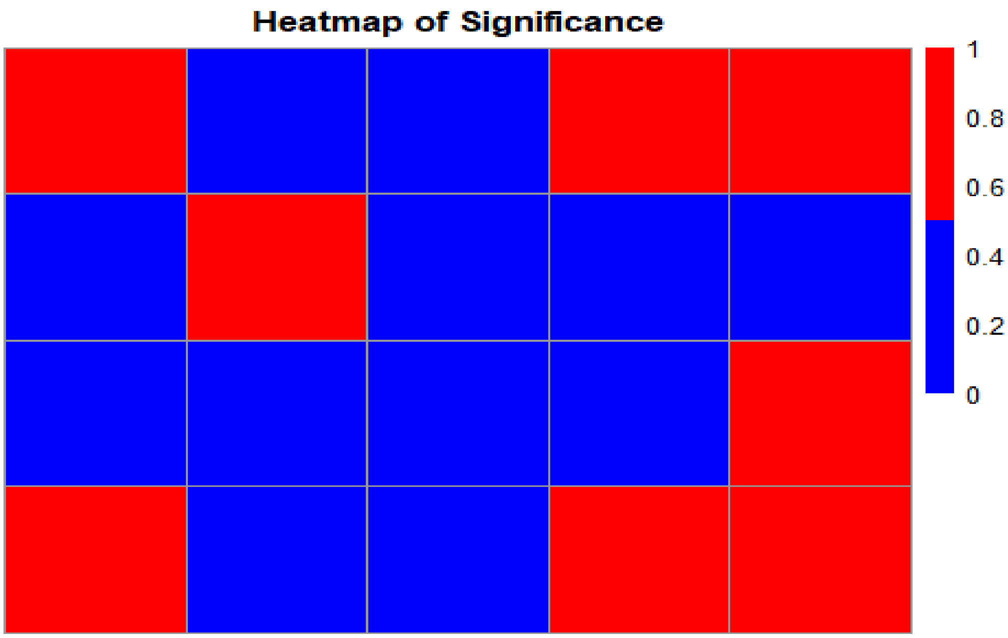


Figure 1: Heat map of the *p-value*-based matrix of the multiple comparisons of the number of observed samples row-wise and column-wise. The red color shows the significant difference between the cell with cells. The blue color represents the non-significant differences.

Table 4: Zone 1 milk samples results tested for the presence of antibiotic residues. The observed data has been presented as n (% out of the total sample in the table) along with the 95% Confidence interval [95% C.I].

Category	B-lactams positive	Tetracycline positive	Both positive	Weak positive	Both negative	*P-value*
Raw milk of cow	3 (7.5%) [19.86-2.58]	0 (0%) [8.76-0]	3 (7.5%) [19.86-2.58]	1 (2.5%) [12.88-0.13]	3 (7.5%) [19.86-2.58]	*0.1039*
Raw milk of buffalo	1 (2.5%) [12.88-0.13]	1 (2.5%) [12.88-0.13]	2 (5%) [16.5-0.89]	0 (0%) [0-0]	6 (15%) [29.07-7.06]	
Processed UHT milk	1 (2.5%) [12.88-0.13]	0 (0%) [8.76-0]	0 (0%) [8.76-0]	0 (0%) [8.76-0]	9 (22.5%) [37.5-12.32]	
Processed pasteurized milk	0 (0%) [8.76-0]	0 (0%) [8.76-0]	2 (5%) [16.5-0.89]	0 (0%) [8.76-0]	8 (20%) [34.76-10.5]	

Table 5: Zone 2 milk samples results tested for the presence of antibiotic residues. The observed data has been presented as n (% out of the total sample in the table) along with the 95% Confidence interval [95% C.I].

Category	β-lactams positive	Tetracyclines positive	Both positive	Weak positive	Both negative	*P-value*
Raw milk of cow	2 (2.5%) [8.66 - 0.44]	0 (0%) [4.58 - 0]	0 (0%) [4.58 - 0]	1 (1.25%) [6.75 - 0.06]	7 (8.75%) [16.98 - 4.3]	*0.6727*
Raw milk of buffalo	0 (0%) [4.58 - 0]	0 (0%) [4.58 - 0]	1 (1.25%) [6.75 - 0.06]	1 (1.25%) [6.75 - 0.06]	8 (10%) [18.51 - 5.15]	
Processed UHT milk	0 (0%) [4.58 - 0]	0 (0%) [4.58 - 0]	0 (0%) [4.58 - 0]	1 (1.25%) [6.75 - 0.06]	9 (11.25%) [20.02 - 6.03]	
Processed pasteurized milk	0 (0%) [4.58 - 0]	0 (0%) [4.58 - 0]	1 (1.25%) [6.75 - 0.06]	0 (0%) [4.58 - 0]	9 (11.25%) [20.02 - 6.03]	

Table 5 presents data on zone 2, focusing on the presence of two types of antibiotics, β-lactams, and tetracyclines, in different categories of milk samples. In the raw milk of cow, 2 samples (2.5%) tested positive for β-lactams, while none of the samples showed the presence of tetracyclines. No samples exhibited dual positivity for both antibiotics, but one sample demonstrated weak positive results for both β-lactams and tetracyclines. On the other hand, 7 samples (8.75%) tested negative for both β-lactams and tetracyclines. The calculated p-value (0.6727) for this category indicates no significant difference between the observed samples in terms of antibiotic presence. In the raw milk of buffalo, no samples tested positive for β-lactams or tetracyclines. One sample (1.25%) demonstrated dual positivity for both antibiotics, while one sample exhibited weak positive results. In this category, 8 samples (10%) tested negative for both β-lactams and tetracyclines. There were no occurrences of β-lactams or tetracyclines positives for processed UHT milk. Weak positive results for both antibiotics were present in one sample (1.25%). Nine (11.25%) of the processed UHT milk samples tested negative for β-lactams and tetracyclines. Similar to processed pasteurized milk, neither β-lactams nor tetracyclines showed any signs of antibiotic positive. One sample (1.25%) did, however, test positive for both antibiotics. Nine samples, or 11.25% of the total, tested negative for β-lactams and tetracyclines. It is clear from the p-values that the observed milk samples do not significantly differ from one another.

Table 6: Zone 3 milk samples results tested for the presence of antibiotic residues. The observed data has been presented as n (% out of the total sample in the table) along with the 95% Confidence interval [95% C.I].

Category	β-lactams positive	Tetracyclines positive	Both positive	Weak positive	Both negative	*P-value*
Raw milk of cow	2 (5%) [16.5 - 0.89]	0 (0%) [8.76 - 0]	2 (5%) [16.5 - 0.89]	2 (5%) [16.5 - 0.89]	4 (10%) [23.05 - 3.96]	*0.03798*
Raw milk of buffalo	2 (5%) [16.5 - 0.89]	1 (2.5%) [12.88 - 0.13]	0 (0%) [8.76 - 0]	2 (5%) [16.5 - 0.89]	5 (12.5%) [26.11 - 5.46]	
Processed UHT milk	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	1 (2.5%) [12.88 - 0.13]	9 (22.5%) [37.5 - 12.32]	
Processed pasteurized milk	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	10 (25%) [40.19 - 14.19]	

Data on milk samples from zone 3 are shown in table 6, with the presence of two types of antibiotic residues β-lactams and tetracyclines in various types of milk being the main focus. The number of positive samples and the proportion of positive samples relative to the total observed samples are shown in the table for each category. The data were analyzed using Fisher's exact test, and the corresponding p-values were given to determine the significance of variations between the observed samples in various categories. Two samples (5%) of cow's raw milk tested positive for β-lactams, although none of the samples contained tetracyclines. Similarly, 2 samples (or 5% of the examined samples) showed dual positive for both β-lactams and tetracyclines. Additionally, 2 samples showed marginally beneficial outcomes for both antibiotics. However, 4 samples of raw cow milk, or 10% of the total observed samples, were negative for tetracyclines and β-lactams. The calculated p-value (0.03798) for this category indicates a significant difference between the observed samples in terms of antibiotic presence. In the raw milk of buffalo, 2 samples (5%) tested positive for β-lactams, and 1 sample (2.5%) showed the presence of tetracyclines. No samples exhibited simultaneous positivity for both antibiotics or weak positive results. In this category, 5 samples (12.5%) tested negative for both β-lactams and tetracyclines. For processed UHT milk, no instances of β-lactams or tetracyclines positivity were found. One sample (2.5%) exhibited weak positive results for both antibiotics. However, the majority of processed UHT milk samples, totaling 9 (22.5%), tested negative for both β-lactams and tetracyclines. Similarly, processed pasteurized milk demonstrated no instances of antibiotic positivity for either β-lactams or tetracyclines. None of the samples exhibited simultaneous positivity for both antibiotics or weak positive results. The majority of the samples, totaling 10 (25%), were negative for both β-lactams and tetracyclines. Based on the p-values, it is evident that there is a significant difference between the observed in the milk samples.

Table 7: Zone 4 milk samples results tested for the presence of antibiotic residues. The observed data has been presented as n (% out of the total sample in the table) along with the 95% Confidence interval [95% C.I].

Category	β-lactams positive	Tetracyclines positive	Both positive	Weak positive	Both negative	*P-value*
Raw milk of cow	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	2 (5%) [16.5 - 0.89]	2 (5%) [16.5 - 0.89]	6 (15%) [29.07 - 7.06]	*0.3408*
Raw milk of buffalo	1 (2.5%) [12.88 - 0.13]	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	2 (5%) [16.5 - 0.89]	7 (17.5%) [31.95 - 8.75]	
Processed UHT milk	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	2 (5%) [16.5 - 0.89]	0 (0%) [8.76 - 0]	8 (20%) [34.76 - 10.5]	
Processed pasteurized milk	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	1 (2.5%) [12.88 - 0.13]	0 (0%) [8.76 - 0]	9 (22.5%) [37.5 - 12.32]	

Table 7 presents data on milk samples from zone 4. In the raw milk of cows, none of the samples tested positive for β-lactams or tetracyclines. However, 2 samples (5%) exhibited dual positivity for both antibiotics, while 2 samples showed weak positive results. On the other hand, the majority of raw cow milk samples, totaling 6 (15% of observed samples), were negative for both β-lactams and tetracyclines. The calculated p-value (0.3408) for this category indicates no significant difference between the observed samples in terms of antibiotic presence. In the raw milk of buffalo, 1 sample (2.5%) tested positive for β-lactams, while none of the samples showed the presence of tetracyclines. No samples exhibited simultaneous positivity for both antibiotics or weak positive results. In this category, 7 samples (17.5%) tested negative for both β-lactams and tetracyclines. For processed UHT milk, none of the samples tested positive for β-lactams or tetracyclines. However, 2 samples (5%) exhibited dual positivity for both antibiotics. The majority of processed UHT milk samples, totaling 8 (20%), tested negative for both β-lactams and tetracyclines. Similarly, processed pasteurized milk demonstrated no instances of antibiotic positivity for either β-lactams or tetracyclines, except for 1 sample (2.5%) that exhibited dual positivity for both antibiotics. The majority of the samples, totaling 9 (22.5%), were negative for both β-lactams and tetracyclines. Based on the p-values, it is evident that there is no significant difference between the observed samples in the milk of different categories.

Table 8 presents data on milk samples from zone 5. In the raw milk of cow, 1 sample (2.5%) tested positive for β-lactams, while none of the samples showed the presence of tetracyclines. Additionally, 1 sample exhibited dual positivity for both antibiotics, and 2 samples showed weak positive results. On the other hand, the majority of raw cow milk samples, totaling 6 (15% of observed samples), were negative for both β-lactams and tetracyclines. The calculated p-value (0.5017) for this category indicates no significant difference between the observed samples in terms of antibiotic presence. In the raw milk of buffalo, no samples tested positive for β-lactams, but 1 sample (2.5%) showed the presence of tetracyclines. No samples exhibited simultaneous positivity for both antibiotics or weak positive results. In this category, 8 samples (20%) tested negative for both β-lactams and tetracyclines. For processed UHT milk, none of the samples tested positive for β-lactams or tetracyclines. However, 1 sample (2.5%) exhibited weak positive results for both antibiotics. The majority of processed UHT milk samples, totaling 9 (22.5%), tested negative for both β-lactams and tetracyclines. Similarly, processed pasteurized milk demonstrated no instances of antibiotic positivity for either β-lactams or tetracyclines, except for 2 samples (5%) that exhibited dual positivity for both antibiotics. The majority of the samples, totaling 8 (20%), were negative for both β-lactams and tetracyclines. Based on the provided p-values, it is evident that there is no significant difference between the observed samples in the milk categories.

Table 9 presents an overview of the zone-wise detection of kit results. In Zone 1, 5 samples (2.5%) tested positive for β-lactams, while 1 sample (0.5%) showed the presence of tetracyclines. Additionally, 7 samples (3.5%) exhibited dual positivity for both antibiotics and 1 sample (0.5%) showed weak positive results for both. The majority of samples, totaling 26 (13%), were negative for both β-lactams and tetracyclines. The estimated p-value (0.4278) for Zone 1 shows that there is no significant difference in antibiotic presence between the observed samples. Two samples (1% of the total) from Zone 2 tested positive for β-lactams, however, none of the samples contained tetracyclines. Three samples (1.5%) had weak positive results for both antibiotics, and 2 samples (1%) demonstrated dual positivity for both. 33 samples, or 16.5% of the total, were negative for both tetracyclines and β-lactams. In Zone 3, 4 samples (2%) tested positive for β-lactams, while 1 sample (0.5%) showed the presence of tetracyclines. Furthermore, 5 samples (2.5%) had weak positive results for both antibiotics, and 2 samples (1%) demonstrated dual positivity for both. 28 samples, or 14% of the total, were negative for tetracyclines and β-lactams. One sample (0.5%) from Zone 4 tested positive for β-lactams, however, none of the samples contained tetracyclines. In addition, 4 samples (2%) had marginally positive findings for both antibiotics, while 5 samples (2.5%) indicated dual positivity for both. Thirty samples, or 15% of the total, were negative for β-lactams and tetracyclines. In Zone 5, 1 sample (0.5%) tested positive for β-lactams, while 1 sample (0.5%) showed the presence of tetracyclines. Furthermore, 4 samples (2%) had weakly positive results for both antibiotics, and 3 samples (1.5%) demonstrated dual positivity for both. 31 samples, or 15.5% of the total, were negative for tetracyclines and β-lactams. According to the given p-value (0.4278), there is no discernible difference between the observed samples in terms of the presence of antibiotics in the different study area zones.

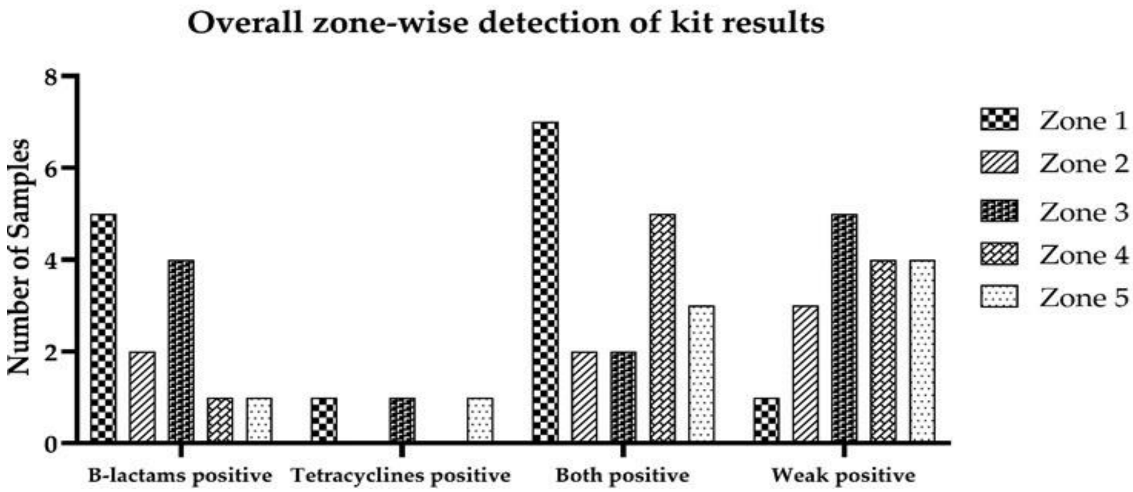
Table 10 provides a comparison of the antibiotic residues found in samples of raw milk from cows and buffaloes and processed milk from UHT and pasteurized milk. β-lactams positive, tetracyclines positive, both positive, Weak positive (both antibiotics weakly positive), and Both negative (both antibiotics negative) are the classifications. The information is presented as n (the number of samples observed in each category) and the proportion of observed values to all observed values in the corresponding category. In the cow raw milk category, 8 samples (2%) tested positive for β-lactams, while none of the samples showed the presence of tetracyclines. Additionally, 8 samples (2%) exhibited dual positivity for both antibiotics, and 8 samples (2%) showed weak positive results for both. The majority of cow raw milk samples, totaling 26 (6.5%), were negative for both β-lactams and tetracyclines. In the buffalo raw milk category, 4 samples (1%) tested positive for β-lactams, while 3 samples (0.75%) showed the presence of tetracyclines. Additionally, 3 samples (0.75%) exhibited dual positivity for both antibiotics, and 6 samples (1.5%) showed weak positive results for both. The majority of buffalo milk samples, totaling 34 (8.5%), were negative for both β-lactams and tetracyclines. Overall, when considering both cow and buffalo samples (Total), 12 samples (3%) tested positive for β-lactams, 3 samples (0.75%) showed the presence of tetracyclines, and 11 samples (2.75%) exhibited dual positivity for both antibiotics. Furthermore, 14 samples (3.5%) showed weak positive results for both antibiotics. The majority of total milk samples, totaling 60 (15%), were negative for both β-lactams and tetracyclines. In the processed milk categories (UHT and Pasteurized), the presence of antibiotics was significantly reduced compared to raw milk. For UHT milk, only 1 sample (0.25%) tested positive for β-lactams, while none of the samples showed the presence of tetracyclines. Additionally, 2 samples (0.5%) exhibited dual positivity for both antibiotics, and 3 samples (0.75%) showed weak positive results for both. The majority of UHT milk samples, totaling 44 (11%), were negative for both β-lactams and tetracyclines. For pasteurized milk, no samples tested positive for β-lactams or tetracyclines. However, 6 samples (1.5%) exhibited dual positivity for both antibiotics and the majority, totaling 44 (11%), were negative for both β-lactams and tetracyclines. The calculated p-value (0.0004998) for the comparison between raw and processed milk categories in terms of β-lactams positive samples indicates a significant difference. The p-value less than 0.05 suggests a significant difference between the observed samples in these categories.

Table 8: Zone 5 milk samples results tested for the presence of antibiotic residues. The observed data has been presented as n (% out of the total sample in the table) along with the 95% Confidence interval [95% C.I].

Category	β-lactams positive	Tetracyclines positive	Both positive	Weak positive	Both negative	*P-value*
Raw milk of cow	1 (2.5%) [12.88 - 0.13]	0 (0%) [8.76 - 0]	1 (2.5%) [12.88 - 0.13]	2 (5%) [16.5 - 0.89]	6 (15%) [29.07 - 7.06]	*0.5017*
Raw milk of buffalo	0 (0%) [8.76 - 0]	1 (2.5%) [12.88 - 0.13]	0 (0%) [8.76 - 0]	1 (2.5%) [12.88 - 0.13]	8 (20%) [34.76 - 10.5]	
Processed UHT milk	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	1 (2.5%) [12.88 - 0.13]	9 (22.5%) [37.5 - 12.32]	
Processed pasteurized milk	0 (0%) [8.76 - 0]	0 (0%) [8.76 - 0]	2 (5%) [16.5 - 0.89]	0 (0%) [8.76 - 0]	8 (20%) [34.76 - 10.5]	

Table 9: Overall zone-wise presence of antibiotics in the milk samples detected through the kit. The observed data has been presented as n (% out of the total sample in the table) along with the 95% Confidence interval [95% C.I]

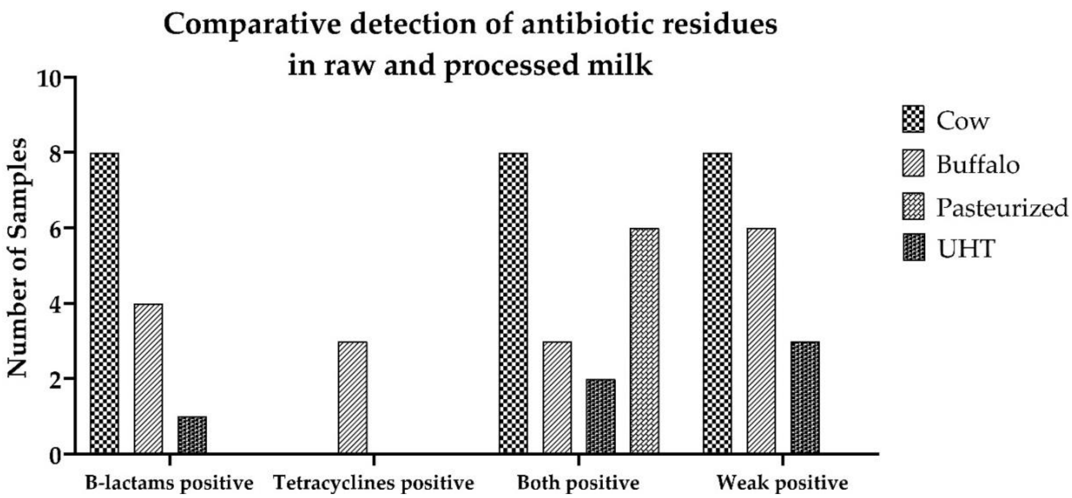
Category	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5	*P-value*
β-lactams positive	5 (2.5%) [5.72 - 1.07]	2 (1%) [3.57 - 0.18]	4 (2%) [5.03 - 0.78]	1 (0.5%) [2.78 - 0.03]	1 (0.5%) [2.78 - 0.03]	
Tetracyclines positive	1 (0.5%) [2.78 - 0.03]	0 (0%) [1.88 - 0]	1 (0.5%) [2.78 - 0.03]	0 (0%) [1.88 - 0]	1 (0.5%) [2.78 - 0.03]	
Both positive	7 (3.5%) [7.05 - 1.71]	2 (1%) [3.57 - 0.18]	2 (1%) [3.57 - 0.18]	5 (2.5%) [5.72 - 1.07]	3 (1.5%) [4.32 - 0.41]	*0.4278*
Weak positive	1 (0.5%) [2.78 - 0.03]	3 (1.5%) [4.32 - 0.41]	5 (2.5%) [5.72 - 1.07]	4 (2%) [5.03 - 0.78]	4 (2%) [5.03 - 0.78]	
Both negative	26 (13%) [18.37 - 9.03]	33 (16.5%) [22.27 - 12]	28 (14%) [19.49 - 9.87]	30 (15%) [20.61 - 10.71]	31 (15.5%) [21.16 - 11.14]	



Graph 1: Graphical representation of the overall results of milk samples screening for antibiotics

Table 10: Comparative detection of antibiotic residues in raw and processed milk. The observed data has been presented as n (% out of the total sample in the table) along with the 95% Confidence interval [95% C.I].

Category	Raw milk			Processed Milk			*P-value*
	Cow Milk	**Buffalo Milk**	**Total**	**UHT**	**Pasteurized**	**Total**	
B-lactams positive	8 (2%) [3.9 - 1.02]	4 (1%) [2.54 - 0.39]	12 (3%) [5.17 - 1.72]	1 (0.25%) [1.4 - 0.01]	0 (0%) [0.95 - 0]	1 (0.25%) [1.4 - 0.01]	
tetracycline positive	0 (0%) [0.95 - 0]	3 (0.75%) [2.18 - 0.2]	3 (0.75%) [2.18 - 0.2]	0 (0%) [0.95 - 0]	0 (0%) [0.95 - 0]	0 (0%) [0 - 0]	
Both positive	8 (2%) [3.9 - 1.02]	3 (0.75%) [2.18 - 0.2]	11 (2.75%) [4.86 - 1.54]	2 (0.5%) [1.8 - 0.09]	6 (1.5%) [3.23 - 0.69]	8 (2%) [3.9 - 1.01]	*0.0005*
Weak positive	8 (2%) [3.9 - 1.02]	6 (1.5%) [3.23 - 0.69]	14 (3.5%) [5.79 - 2.1]	3 (0.75%) [2.18 - 0.2]	0 (0%) [0.95 - 0]	3 (0.75%) [2.18 - 0.20]	
Both negative	26 (6.5%) [9.35 - 4.47]	34 (8.5%) [11.64 - 6.15]	60 (15%) [18.83 - 11.83]	44 (11%) [14.45 - 8.3]	44 (11%) [14.45 - 8.3]	88 (22%) [26.32 - 18.21]	



Graph 2: Graphical representation of the comparative detection of antibiotic residues in raw and processed milk

It was observed that for the β-lactams category, raw milk from cows exhibited a p-value of 0.009, indicating a significant difference in the number of samples identified compared to other milk categories for this antibiotic. Similarly, raw milk from buffalos also displayed a significantly low p-value of 0.551. Table 4.10 shows a p-value based matrix of the multiple comparisons of many observed samples row-wise and column-wise.

Table 11: Shows *p-value* based matrix of the multiple comparison of the number of observed samples row-wise and column-wise.

Kit Results	Raw milk			Processed milk		
	Cow Milk	Buffalo Milk	Total	UHT	Pasteurized	Total
β-lactams positive	*0.009*	*0.551*	***0.017***	*0.228*	*0.059*	***0.008***
Tetracyclines positive	*1*	*0.028*	***0.168***	*1*	*1*	***0.344***
Both positive	*0.118*	*0.604*	***0.557***	*0.201*	*0.451*	***0.694***
Weak positive	*0.055*	*0.412*	***0.036***	*0.785*	*0.013*	***0.022***
Both negative	*0.0004*	*0.305*	***0.0004***	*0.015*	*0.015*	***0.0002***

However, both processed milk categories had relatively low absolute counts for β-lactams positive samples, with 0.228 for UHT milk and 0.059 for pasteurized milk. Regarding tetracyclines, all categories of processed milk showed a p-value of 1, suggesting no significant difference in the number of samples identified for this antibiotic. On the other hand, raw buffalo milk had a notably low p-value of 0.028, indicating a significant difference in the number of samples identified compared to the other milk categories. When considering both antibiotics together, the category of both positive samples showed varying results. Raw buffalo milk had a higher p-value of 0.604 compared to raw cow milk, indicating a significant difference in the number of samples found. The p-values were lower for processed milk categories, with 0.201 for UHT milk and 0.451 for pasteurized milk, indicating notable variations in the number of samples found to both antibiotics. Processed UHT milk exhibited a relatively high p-value of 0.785 for weak positive signals, indicating that there was no significant difference in the number of samples found compared to other milk categories. Raw cow milk, on the other hand, displayed a p-value of 0.055, which revealed a significant difference in the number of weakly positive samples. UHT milk had a p-value of 0.785 and pasteurized milk had quite low p-values of 0.013, respectively, for processed milk categories, indicating substantial differences in the number of weak positive results compared to other milk kinds. Last but not least, raw cow milk had a p-value of 0.0004 in the case of both negative samples (absence of both antibiotics), showing a significant difference in the number of samples discovered compared to the other milk categories. The p-value for raw buffalo milk was strikingly high (0.305), indicating that there was no discernible difference. Both UHT and pasteurized milk categories for processed milk exhibited extremely low p-values of 0.015, demonstrating significant differences in the number of samples noticed when both antibiotics were absent.

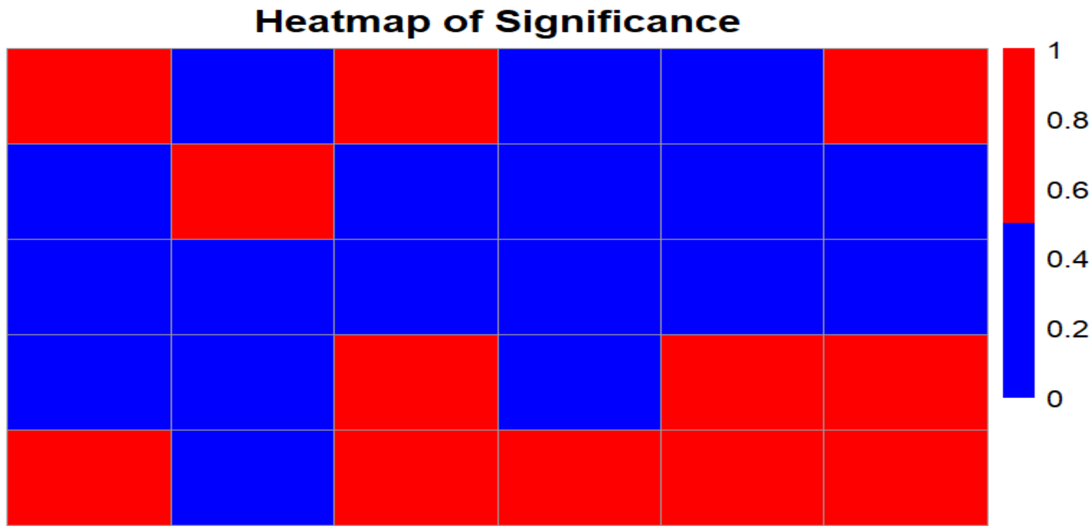


Figure 2: Heat map of the *p-value*-based matrix of the multiple comparisons of the number of observed samples row-wise and column-wise. The red color shows the significant difference between the cell with cells. The blue color represents the non-significant differences

DISCUSSION

The current study investigated the antibiotic residues (β-lactam and tetracycline) in processed and raw milk which possess detrimental effects on both human and animal health. However, inappropriate and imprudent antibiotic use, as well as a lack of understanding of the compulsory withdrawal period might result in unwanted antibiotic residues appearing in the milk of treated animals (Kumar *et al*. 2021). In this current study among all 4 categories of milk collected from 5 zones of Lahore, 6.5% (13/200) of milk samples were positive for only beta-lactams, 1.5% (3/200) milk samples were positive for only tetracyclines, 9.5% (19/200) milk samples were positive for beta-lactams and tetracyclines both, 8.5% (17/200) milk samples were weakly positive for beta-lactams and tetracyclines both, and 74% (148/200) milk samples were negative for beta-lactams and tetracyclines both.

A similar study was conducted by Chowdhury S *et al*. 2015. at Raozan Upazila and Chittagong Metropolitan area of Chittagong district in which the residues of three antibiotics tetracycline, amoxicillin, and ciprofloxacin were detected. Of 200 milk samples of local cow milk and commercial cow milk, 12% were positive for tetracycline in local cow milk and 23% were positive for commercial cow milk. Similarly, among the 200 milk samples analyzed, 14% were positive for Amoxicillin in local cow milk and 38% were found positive for commercial cow milk. Residues of ciprofloxacin were dominant in commercial cow milk which was 17% than in local cow milk which was 8%. In another study by Moghadam MM *et al*. 2016, in a total of 251 milk samples containing 143 commercial pasteurized milk packets, 84 raw milk, and 24 pasteurized milk, 189 samples (75.2%) were found negative and 62 (24.8%) were positive. 41 samples (28.7%) of the commercial pasteurized milk packet, 18 samples (21.4%) of the raw milk, and 3 samples (12.5%) of the pasteurized milk were positive for the presence of antibiotic residues. A study was organized in Kenya by Ahlberg S *et al*. 2016 in which they analyzed a total of 480 milk samples they determined 114 (24%) samples were positive, 71 (15%) unclear, and 295 (61%) negative. 62 samples were examined further using the Trisensor test that is unique to each group. 15/62 (24%) were in favor. This revealed that 5% of the 480 samples, or 9% of them, were estimated to have included beta-lactams, 2.5% sulfonamides, and 0.6% tetracyclines, according to the Trisensor test results. Brady MS and Katz SE (1988) found that 63% of milk samples in New Jersey had one or more antibiotic residues, 27% had two residues, and 11% had three or more residues. The most common residues found were those from tetracyclines and sulfonamides. According to Gaurav *et al*. 2014, in a total of 133 cattle milk samples, 18 samples were found to contain tetracycline residues in 5 districts of Punjab state, India. In the Castilla La Mancha region of Spain, Yamaki M. *et al*. 2004 reported that 1.7% of samples were positive for antibiotic residues in the total of 2686 raw milk samples of ewe. Kaya SE and Filazi A (2010) showed 1.25% of milk sample positive for antibiotic residues in the total of 240 milk samples, which contains raw milk and pasteurized milk products sold in Ankara Turkey. Aalipour F *et al.* in 2013 reported 37 samples (19.8%) with antibiotic residues above the European Union Maximum Residues Limits (EU-MRLs), and 28 samples (14.97%) at the concentrations below the EU-MRLs of 187 commercial milk samples, including 154 pasteurized and 33 sterilized milk samples. Brown *et al.* 2020 quantified 7.4% β-lactam residues and 3.2% tetracycline residues of 95 milk samples (74 pasteurized and 21 unpasteurized) collected from shops, street vendors, or vending machines in Nairobi, Kenya.

The goal of the current study is to compare the antibiotic residues of tetracyclines and beta-lactams in raw and processed milk from various locations within the same city (Lahore). The current investigation was carried out in 5 zones of Lahore. Out of 40 samples of all 4 categories collected from each zone. In Zone 1, 5 samples (2.5%) tested positive for β-lactams, while 1 sample (0.5%) showed the presence of tetracyclines. Additionally, 7 samples (3.5%) exhibited dual positivity for both antibiotics and 1 sample (0.5%) showed weak positive results for both. The majority of samples, totaling 26 (13%), were negative for both β-lactams and tetracyclines. The estimated p-value (0.4278) for Zone 1 shows that there is no significant difference in antibiotic presence between the observed samples. Two samples (1% of the total) from Zone 2 tested positive for β-lactams, however, none of the samples contained tetracyclines. Three samples (1.5%) had weak positive results for both antibiotics, and 2 samples (1%) demonstrated dual positivity for both. 33 samples, or 16.5% of the total, were negative for both tetracyclines and β-lactams. In Zone 3, 4 samples (2%) tested positive for β-lactams, while 1 sample (0.5%) showed the presence of tetracyclines. Furthermore, 5 samples (2.5%) had weak positive results for both antibiotics, and 2 samples (1%) demonstrated dual positivity for both. 28 samples, or 14% of the total, were negative for tetracyclines and β-lactams. One sample (0.5%) from Zone 4 tested positive for β-lactams, however, none of the samples contained tetracyclines. In addition, 4 samples (2%) had marginally positive findings for both antibiotics, while 5 samples (2.5%) indicated dual positivity for both. Thirty samples, or 15% of the total, were negative for β-lactams and tetracyclines. In Zone 5, 1 sample (0.5%) tested positive for β-lactams, while 1 sample (0.5%) showed the presence of tetracyclines. Furthermore, 4 samples (2%) had weakly positive results for both antibiotics, and 3 samples (1.5%) demonstrated dual positivity for both. 31 samples, or 15.5% of the total, were negative for tetracyclines and β-lactams. According to the given p-value (0.4278), there is no discernible difference between the observed samples in terms of the presence of antibiotics in the different study area zones.

In the comparison of the antibiotic residues found in samples of raw and processed milk, in the cow raw milk category, 8 samples (2%) tested positive for β-lactams, while none of the samples showed the presence of tetracyclines. Additionally, 8 samples (2%) exhibited dual positivity for both antibiotics, and 8 samples (2%) showed weak positive results for both. The majority of cow milk samples, totaling 26 (6.5%), were negative for both β-lactams and tetracyclines. In the buffalo raw milk category, 4 samples (1%) tested positive for β-lactams, while 3 samples (0.75%) showed the presence of tetracyclines. Additionally, 3 samples (0.75%) exhibited dual positivity for both antibiotics, and 6 samples (1.5%) showed weak positive results for both. The majority of buffalo raw milk samples, totaling 34 (8.5%), were negative for both β-lactams and tetracyclines. In the processed milk categories (UHT and Pasteurized), the presence of antibiotics was significantly reduced compared to raw milk. For UHT milk, only 1 sample (0.25%) tested positive for β-lactams, while none of the samples showed the presence of tetracyclines. Additionally, 2 samples (0.5%) exhibited dual positivity for both antibiotics, and 3 samples (0.75%) showed weak positive results for both. The majority of UHT milk samples, totaling 44 (11%), were negative for both β-lactams and tetracyclines. For pasteurized milk, no samples tested positive for β-lactams or tetracyclines. However, 6 samples (1.5%) exhibited dual positivity for both antibiotics and the majority, totaling 44 (11%), were negative for both β-lactams and tetracyclines. The calculated p-value (0.0004998) for the comparison between cow and buffalo categories in terms of β-lactams positive samples indicates a significant difference. The p-value less than 0.05 suggests a significant difference between the observed samples in these categories.

In this study, the antibiotic residues are detected using bioeasy milk antibiotic rapid test for β-lactams and tetracyclines in the milk kit method. This kit is based on the technique of colloidal gold immunochromatography technology also known as Lateral flow dipstick immunoassay. Wei Z and Wang J in 2011 used a voltammetric electronic tongue (VE-tongue) to identify antibiotic residues in bovine milk. In 2020, Teixeira RC *et al*. studied the occurrence of β-lactam antibiotics in bovine milk using vibrational spectroscopy and DFT theoretical simulations. In another study thin-layer chromatography (TLC) and ultra-high-performance liquid chromatography (UHPLC) techniques were used for the determination of veterinary antibiotic residues (Rahman MS *et al*. 2021). In 2011, Fernandez F *et al*. utilized the SPReeta Evaluation Kit SPR3, a cheap and portable surface plasmon resonance (SPR) sensor, to create a biosensor for the detection of fluoroquinolone antibiotics (FQs) and to verify the effectiveness of the biosensor by analysing FQ residues in milk samples. A study was carried out in Kenya in which all samples were analyzed with the Delvotest® screening test (Ahlberg S *et al*. 2016). In this study, the kit method is used for the determination of β-lactams and tetracyclines residues which is based on the technique of colloidal gold immunochromatography technology also known as Lateral flow dipstick immunoassay because this kit is easy to use and takes minimum time to perform one test.

According to the findings of the current investigation, milk samples marketed in Lahore had antibiotic residues. Most often found antibiotic residues were β-lactams and tetracyclines. These residues were more in the raw milk as compared to the processed milk.

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