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Research Article

Detection of Antibiotic Residues of Penicillin and Oxytetracycline in Milk

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Authors' Contributions
MAR conducted research and wrote the manuscript. AZD supervised the research. MHS and KA helped in research. AZD, MHS and KA reviewed the article. MMA, NR and KHA facilitated in lab procedures. KHA collected the samples. NR and KHA compiled data.

Keywords
Food safety, Antibiotic residues, One health, Animal health

Abstract | Nevertheless, the discovery and increased production of pharmaceuticals has lead to substantial use of pharmaceutical products that include antibiotics and steroidal hormones. The day by day increase in human population across the globe is leading to the increased need of food production. Due to the excessive use of common antibiotics i.e. penicillin and oxytetracycline, the antibiotic (ABs) residues remain in livestock products like milk of buffalo and cow. It leads to antibiotic resistance in human beings as well as animals and thus lead to the problem for one health (OH). Following were the objectives of the current study. Detection of commonly used antibiotics (penicillin and oxytetracycline) in (i) raw milk of buffalo and cow collected open market, (ii) UHT milk, (iii) pasteurized milk, (iv) bulk tank milk and (v) gawala's (milk man) milk in Lahore. Kit detection and HPLC methods were used to identify ABs residues. The samples were collected from Lahore. Raw milk of buffalo and cow collected from the open market have shown 23.5% as the highest positive and UHT milk as the lowest positive 8.5%. Thus, it is an indispensable question to be further explored prospectively.

Novelty Statement | The detection of antibiotic residues including penicillin and oxytetracycline in six different categories of milk were categorically studied for the first time in Lahore with the help of bioassay rapid test and HPLC.

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Introduction

Nevertheless, with the discovery and increased production of pharmaceuticals, there has been a considerable dependence upon the use of pharmaceuticals which includes antibiotics (ABs), steroidal hormones and plenty of other medicines. ABs are widely for disease treatment and prevention in livestock and veterinary spheres during the contemporary epoch. The emergence and discovery of animal and environmental health has revealed endanger to one health (OH) and requires the exigent attention and active response from all the countries

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(Fatoki *et al*., 2018). The study has delineated that the clinical waste product systems add fuel to the fire of antibiotic resistance (Voigt *et al*., 2019). ABs are extensively used in human beings as well as veterinary sectors (Lu *et al*., 2019).

After the inspections of livestock economics, the TFDA could not discover the residues of medicines that were declared illegal by the government. The detection of alternative medicines have shown decreasing trend (Lee *et al*., 2017). Milk quality refers to a variety of characteristics which augment the acceptability of milk and milk products. These parameters may inculcate chemical, physical, technological, bacteriological and aesthetic characteristics. Milk safety refers to the state whereby milk is safe for human consumption. In this review, antibiotic use in the foodstuffs and their effects on the human health were discussed (Bacanli and Basaran, 2019). Poor milk safety poses a persistent hazard to public health. The results of these studies indicate that there is a dire need to improve the quality and safety of milk (Nyokabi *et al*., 2021). Nevertheless, the improper use of veterinary drugs such as ignoring the required withdrawal periods results into the presence of drug residues in animals. It has adverse implications on food safety. Thus, this has serious implications on public health and eventually one health (Pugajeva *et al*., 2019). This can prove very significant in order to protect human beings by controlling the undesirable residues in livestock products (Kyriakides *et al*., 2020). As in comparison of techniques, chromatographic technique has shown the highest results i.e. 115 (51.34%) for detection of antibiotic residues in milk. There must be quality control of this kind of nutritious food. The intake of excessive amounts of these antimicrobial substances in any form like injectables is of tremendous concern, as they may have serious harmful implications for human health like allergic and toxic reactions along with increasing bacterial resistance (Teixeira *et al*., 2020).

According to the contemporary emerging phenomenon of OH, the health of human beings is closely connected with animal health and including the environment. OH approach focuses on management of health in both human and animals (Acharya *et al*., 2020). The term one health is related to the integration of separately considered subjects of humans, animals and their environment. A variety of departments and agencies play their role from public health perspective in different countries of the world. OH methods emphasize on the data sharing specially to inculcate the full integration of the public health (Allard *et al*., 2020). The initiative of "One World-One Health" endeavours and strife to rapidly figure out emerging or re-emerging human and animal diseases and to prevent environmental challenges like deforestation (Miró and López-Vélez, 2018). It is critical that all sectors collaborate in these endeavours including the policies formulation and implementation through legislation (Kardjadj and Ben-Mahdi, 2019). As a matter of fact, since there is a tremendous proportions of the administered pharmaceuticals, therefore, it raises a question about the residues of these drugs of divergent nature and chemical composition in a variety of food items like milk, beef and mutton (Fatoki *et al*., 2018). The eighty percent (80 %) of the animals are currently being treated with veterinary drugs which are engaged in food production (Bacanli and Başaran, 2019). Prospectively, it was recommended by the scholars that the research should focus on policy making theme in order to provide clear evidence on benefits of using the OH approach (Dos *et al*., 2019). The scientists need to take advantage from all the experts from a variety of sectors and it is indispensable to work in collaboration for a single aim of OH achievement through food safety (Balkhy *et al*., 2018). In year 2010, the World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE) engaged in a tripartite collaboration to coordinate for OH (Humboldt-Dachroeden *et al*., 2020). There is a need to robust the importance of OH and to consider it as a joint venture for all kinds of health (Davis and Sharp, 2020). It is still unclear whether sufficient education and training are being provided to the veterinary students regarding OH and its importance (Franco-Martínez *et al*., 2020). Massive Open Online Courses (MOOC) programs were initiated. Thus, this has emphasized on the need for scientific research in the realm of OH through interdisciplinary international collaborations for the proper use of the veterinary drugs in livestock sector (Bolon *et al*., 2020). Inculcation of OH concepts into medical and veterinary clinical program will normalize the OH practice. It will equip General Medical Practitioners (GMPs) and veterinarians to play their crucial roles as a 'frontline' responders (Steele *et al*., 2019).

Materials and Methods

Selection of area for sampling

The samples were collected from different areas and localities area-wise i.e. Valencia, Johar Town, WAPDA Town, Thokar Canal, Wahdat Road, Niqsha, Mazang, Iqbal Town, Bheiky waal, Scheme Mor, Multan Road, Bakar Mandi, Muslim Town Moor, Liton Road, Shadman, Mall Road, Gulberg, Model Town, Kalma Chowk, Qasoor road, Kahna Kacha, Cantonment, Garhi Shahu, Railway Station, Mughal Pura, Bhagwan Pura, Islam Pura, Sannat Nagar, walled city, Darbar Bhaati, Lahore district of Punjab province, Pakistan.

Sampling parameters

The experimental sources included raw milk of buffalo and cow collected open market, UHT milk, pasteurized milk, bulk tank milk and gawala's (milkman) milk from Lahore district of Punjab province, Pakistan.

Sample types

The sample types were (i) milk of buffalo and cow collected open market, (ii)UHT milk, (iii)pasteurized milk, (iv) bulk tank milk and (v) gawala's (milkman) milk.

Sample size

The details of sample size are given as per following (Table 1).

Table 1: Number of samples.

Category	Total samples
(i) Raw milk of buffalo and cow collected open market	200
(ii) UHT milk,	200
(iii) Pasteurized milk,	200
(iv) Bulk tank milk and	200
(v) Gawala's (milk man) sold milk	200
Total	1000

Samples collection and preservation

Milk samples were collected in falcon tubes and were transported to the laboratory in ice boxes. Those were kept in refrigerator for cooling purpose before processing.

Sample processing and testing method

A few methods are available according to the cited literature, however, the researcher has chosen bioassay rapid test kit method (Kit ID: YRM1007-401) and High-performance liquid chromatography (HPLC) for the detection and quantification of beta-lactams and tetracyclines (Lee *et al.*, 2017). Nevertheless, the recent sensitive techniques have been developed, the antibiotics residue quantification in foods remain a challenging task due to the complexity of the samples and the trace concentration level. High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) is one of the best sensitive technique among many (Lu *et al.*, 2019). The quantification of antibiotic residues of positive samples was performed under HPLC. The samples were processed according to the standard procedures available with Kit and HPLC apparatus.

Kit methodology

In the contemporary epoch of science, the commonly used detection methods are HPLC and LC-MS/MS and ELISA for the detection of antibiotics residues in milk, meat and other animal food products. Despite the fact that these methods are quite sensitive but still these are laborious, exhaustive and costly. Biosensors are emerging analytical tools for simple, on-site, low cost, specific and sensitive detection of antibiotics (Majdinasab *et al.*, 2020). The scholars have suggested that for monitoring purpose, the law enforcement agencies and administration must use a variety of reliable and accurate analytical methods to detect antimicrobial residues in milk such as HPLC; Gas chromatography mass spectrometry (GC-MS) and rapid kit tests (Teixeira *et al.*, 2020).

Statistical analysis

The samples are being presented as mean ± standard deviation. The obtained data was subjected to analysis by using Chi-Square and ANOVA statistical analyses techniques through SPSS 20.20 software.

Results and Discussion

The samples were earlier undergone through bioassay rapid test penicillin and oxytetracycline in milk kit method (Kit ID: YRM1007-401) and later on, the positive samples were passed through to detect quantity of antibiotic residues present in samples as per standard operational procedure. The results delineated were as per following (Table 2).

Table 2: Overall summary of milk samples.

Category	Positive	Positive %	Negative	Negative %
(i) Raw milk of buffalo and cow collected open market	47	23.5	153	76.5
(ii) UHT milk	17	8.5	183	91.5
(iii) Pasteurized milk	28	14	172	86
(iv) Bulk tank milk	41	20.5	159	79.5
(v) Gawala's (milk man) sold milk	43	21.5	157	78.5

Raw milk of buffalo and cow collected open market 23.5 %, gawala's (milk man) milk 21.5%, bulk tank milk 20.5%, pasteurized milk 14 % and UHT milk 8.5% samples were respectively positive from the highest 23.5 % to the lowest 8.5%. This is an alarming situation in a metropolitan city of Pakistan.

Table 3: Chi-square based overall summary of milk samples Chi-Square tests.

	Value	df	Asymp. Sig. (2-sided)
Pearson chi-square	21.265[a]	4	0.0002805
Likelihood ratio	23.032	4	.000
Linear by linear association	.882	1	.348
N of valid cases	1000		

The samples are being presented as mean ± standard deviation. The obtained data was subjected to analysis by using Pearson Chi-Square through SPSS 20.20. The p-value obtained was 0.0002805 for the aforementioned 5 categories of milk only for the positive samples which have been quantified (Table 3). The p-value obtained via ANOVA

Table 4: Delineating penicillin and oxytetracycline in milk samples µg/L.

Drugs in milk	Raw milk	UHT milk	Pasteurized milk	Bulk tank milk	Gawala's milk
Highest penicillin	13.93	13.95	13.94	13.66	13.85
Lowest penicillin	6.77	7.75	8.75	7.19	7.79
Average penicillin	10.27	10.85	10.99	10.68	10.53
Highest oxytetracycline	310.39	313.13	311.01	311.74	308.87
Lowest oxytetracycline	181.81	198.32	183.97	194.47	187.82
Average oxytetracycline	241.47	246.08	246.67	257.94	237.33
Highest both penicillin and oxytetracycline	11.38 and 279.36	10.44 and 266.61	11.88 and 273.84	11.97 and 286.06	10.98 and 292.21
Lowest both penicillin and oxytetracycline	9.33 and 187.33	10.44 and 266.61	10.34 and 251.54	8.66 and 221.74	7.37 and 187.67
Average both penicillin and oxytetracycline	10.48 and 238.55	10.44 and 266.61	11.11 and 262.69	10.05 and 245.41	9.69 and 251.565

was 0.0003 for these 5 categories of milk. A p-value less than 0.05 or equal to 0.05 is statistically significant at a confidence interval of 95%. It indicates strong evidence against the availability of null hypothesis. It depicts that significant difference of antibiotic residues have been figured out among all the categories of milk. In simple words, it means that the positive samples had different quantities in different categories of milk.

The highest penicillin was found in a sample of UHT as 13.95 µg/L or ng/ml, lowest penicillin was found in a sample of raw milk as 6.77 µg/L, average highest penicillin was found in a sample of pasteurized milk as 10.99 µg/L, highest oxytetracycline was observed in a sample of UHT milk as 313.13 µg/L, lowest oxytetracycline was figured out in a sample of as raw milk as 181.81µg/L, average highest oxytetracycline was observed in samples of bulk-tank milk 257.94 µg/L, highest both penicillin and oxytetracycline were figured out in bulk-tank milk as 11.97 µg/L or ng/ml penicillin and 292.21 µg/L as oxytetracycline in gawala's milk respectively. The lowest both penicillin and oxytetracycline simultaneously were figured out in gawala's milk as 7.37 µg/L penicillin and 187.33 µg/L as oxytetracycline in raw milk respectively. The highest average for both penicillin and oxytetracycline simultaneously was measured as penicillin in pasteurized milk as 11.11 µg/L and oxytetracycline as 266.61 µg/L in raw milk respectively (Table 4).

The results regarding penicillin positive samples were: raw milk of buffalo and cow collected open market 11.5%, gawala's (milk man) milk 10.5%, bulk tank milk 9%, then pasteurized milk 7 % and UHT milk 4.5% samples were respectively positive (Table 5).

The p-value obtained was 0.749 against penicillin. It depicts that insignificant difference have been figured out against penicillin in different categories of milk. In simple words, it means that the positive samples had similar quantities in different categories (Table 6).

Table 5: Penicillin positive milk samples and percentages.

Category	Penicillin/ Total (+ve)	Positive %age
(i) Raw milk of buffalo and cow collected open market	23/200	11.5
(ii) UHT milk	09/200	4.5
(iii) Pasteurized milk	14/200	7
(iv) Bulk tank milk	18/200	9
(v) Gawala's (milk man) sold milk	21/200	10.5
Total	85/1000	8.5

Table 6: ANOVA delineating positive milk samples against penicillin

Mean	10.39	10.29	10	10.5	11.01
Standard deviation	1.766	2.17	1.83	1.7	1.49
F value	0.48				
p value	0.749				

The results regarding oxytetracycline positive samples were: raw milk of buffalo and cow collected open market 10.5%, gawala's (milk man) milk 9%, bulk tank milk 8.5%, pasteurized milk 6% and UHT milk 3.5% samples were respectively positive (Table 7).

Table 7: Oxytetracycline positive milk samples revealing percentage.

Category	Oxytetracycline (+ve)	Positive %age
(i) Raw milk of buffalo and cow collected open market	21	10.5
(ii) UHT milk	07	3.5
(iii) Pasteurized milk	12	6
(iv) Bulk tank milk	17	8.5
(v) Gawala's (milk man) sold milk	18	9

The p-value obtained through ANOVA was 0.684 for the quantified samples against oxytetracycline. It depicted insignificant difference among all of the aforementioned categories for oxytetracycline. In simple words, it means that the positive samples had similar quantities in different categories of milk. As a matter of fact, difference of percentage of positive samples of penicillin was also meager among all these categories (Table 8). The p-value obtained through chi-square was 0.066 against oxytetracycline which has depicted insignificant difference (Table 9).

Table 8: ANOVA depicting positive samples percentage categorically against oxytetracycline.

Mean	239.92	241.1	248.65	254.67	248.96
Standard deviation	38.5	39.39	45.35	34.4	35.92
F value	0.57				
p value	0.684				

Table 9: Chi-square depicting positive samples percentage categorically against oxytetracycline Chi-Square tests

	Value	df	Asymp. Sig. (2-sided)
Pearson chi-square	8.793[a]	4	.066
Likelihood ratio	9.582	4	.048
Linear by linear association	.115	1	.734
N of valid cases	1000		

The results regarding both of the ABs i.e. penicillin and oxytetracycline simultaneously found in positive samples were: bulk tank milk 3%, gawala's (milk man) milk 2%, raw milk of buffalo and cow collected from open market 1.5%, pasteurized milk 1%, UHT milk 0.5% samples were respectively positive (Table 10).

Table 10: Both penicillin and oxytetracycline simultaneously positive samples.

Category	Both Penicillin and Oxytetra-cycline (+ve)	Positive % age
(i)Raw milk of buffalo and cow collected open market	3	1.5
(ii) UHT milk	1	0.5
(iii) Pasteurized milk	2	1
(iv) Bulk tank milk	6	3
(v) Gawala's (milk man) sold milk	4	2

The p-value obtained through chi-square was 0.319 against both penicillin and oxytetracycline ABs residues detected simultaneously. It has depicted that insignificant difference have been figured out among all of the aforementioned categories for both penicillin and oxytetracycline ABs residues detected simultaneously (Table 11). A total of 1000 samples of milk were tested out of which 156 were figured out above MRL (Minimum Residual Level) against penicillin and oxytetracycline, making 15.6 % positive and 84.4% negative (Table 12).

Table 11: Chi-square revealing both penicillin and oxytetracycline positive milk samples Chi-Square tests.

	Value	df	Asymp. Sig. (2-sided)
Pearson chi-square	4.700[a]	4	.319
Likelihood ratio	4.810	4	.307
Linear by linear association	1.555	1	.212
N of valid cases	1000		

Table 12: Overall summary of milk samples.

Category	Total samples	Positive	% age	Negative	% age
Milk	1000	156	15.6	844	84.4

Generally, the exposure of human to antibiotics primarily derives from their clinical use and residues in drinking water and food (Wang *et al*., 2017). The limit of detection, defined as a signal to noise ratio of 3, ranged from from 0.2 to 5.0ng/ml of for milk. The recoveries in spiked milk at a concentration of 40 ng/ml ranged from 76.2 to 113.7% with the relative standard deviations varying between 7.2 and 15.8%. One β-lactam, was detected in milk with a concentration of 6.78 ng/ml (Wang *et al*., 2017). Antibiotics (ABs) may cause harmful residual effects, to reduce this, withdrawal periods should be discovered. The staunch control of those residues in food of animal based food is indispensable to boost human health (Menkem *et al*., 2018). The antibiotic category called antimicrobial drugs (AMDs) are widely used for the treatment and control of diseases in animals. Such kind of high routine practices could result into trace amounts of those medicines in livestock products like milk and meat. They pose grave threats to the human health and eventually to the one health. The review seeks to supply with the outline of trends in sample preparation for the identification of antibacterial drug residues in foodstuffs (Rodríguez *et al*., 2018).

A study was conducted throughout the years 2011-2015 in Taiwan. A project was launched by the TFDA that evaluated a large number of samples obtained from livestock. Over the past five (5) years, there had been thirty four (34) samples that were not upto the SOPs, rules and regulations. Those samples contained residues of medicines like beta-agonists, chloramphen-icols, beta-lactam antibiotics, bactericidal medicines, enrofloxacin, and lincomycin. After the inspections of livestock economics, the TFDA could not discover the residues of medications that were declared illegal by the government. The detection of alternative medicines have shown decreasing trend annually (Lee *et al*., 2017). The scholars have discovered that the majority of

Links Researchers

antibiotics (ABs) bind to solid manure particles. This has supported new insights to in-house multi-residue LC-MS/MS technique for analysis (Jansen *et al*., 2019). The researchers have analysed and claimed that their review paper presented the attainments and progresses for antibiotic residues detection via optical strategies (Wang *et al*., 2018). According to the research scholars, the target of their research study was to gauge the prevalence of antmicrobial residues in raw milk in Kosovo during 2009 to 2010. Within the study, milk samples were collected, and qualitatively screened with two (2) completely different tests, the Delvotest SP assay associated an enzyme-linked receptor-binding assay (SNAP). The concentrations varied between a pair of 1 mg/kg and 1.973 mg/kg. Out of the positive samples, seventeen (17) exceeded residue levels for one or a lot of beta-lactam drugs (Rama *et al*., 2017). The study evaluated the presence and distribution of chemical residues. The samples were analyzed by LC-MS/MS and GC–MS/ MS with a good scope of 322 chemical residues. The antibiotic residues were detected at various levels (Lozano *et al*., 2019). In this study, three (3) screenings were conducted through the technique of Liquid Chromatography tandem-Mass High Resolution Spectrometry (LC-HRMS) were developed and were used to evaluate the antimicrobial substance in 254 raw bovine milk samples. In 30 samples Lincomycin residues were observed as 11.8% frequency and 17.29 ppb as mean value. Three samples have shown oxytetracycline respectively at 15.05, 0.82 and 1.59 ppb and two (02) cefapirin and spyramycin at trace level (Chiesa *et al*., 2020).

The restricted accessibility of medication registered for farm animals let the veterinarians go for off-label treatments which may cause antibiotic residues in the products. Proper elimination time may decrease the chances (Quintanilla *et al*., 2018). The core objective is promotion of interdisciplinary teamwork among a variety of domains of study to attain optimal health for humans, animals and the environment. It is critical that all sectors collaborate in these endeavours including the policies formulation and implementation through legislation (Kardjadj and Ben-Mahdi, 2019). Poor milk safety poses a persistent hazard to public health. Milk safety that simply means the milk is safe for human consumption especially containing tolerable limits of antibiotics residues. The main aim and theme of the studies was to probe the variation in raw milk quality. The results of these studies indicate that there is a dire need to improve the quality and safety of milk (Nyokabi *et al*., 2021). Research analyses should be conducted jointly with regulatory agencies to develop policy and bar on abuse of ABs (Rodrigues *et al*., 2017).

Conclusion and Recommendations

The data pertaining to the said used antibiotics has delineated presence of antibiotic residues in raw milk of cow and buffalo, UHT, pasteurized milk, bulk tank milk, gawala's milk in open markets of Lahore.

The objectives of the current studies have been achieved as per following:

- The analyzed data has shown implications of antibiotic residues on national food safety and one-health due to the excessive use of antibiotic use in livestock.
- The baseline data is helpful to suggest pragmatic way-forward for concerned quarter of legislative, judiciary, prescription policy-makers and the stakeholders in the light research data.
- Extension article has been published in national dailies pertaining to general public awareness regarding antibiotic residues in milk.

Since many of the research scholars have delineated the issue, thus, livestock products being consumed by the humans may contain residues of active pharmaceuticals and lead to antibiotic resistance in the humans. Eventually it has become a challenge to the triad of one health i.e. healthy animals, healthy humans and healthy environment. Thus, this makes it an indispensable question to be further explored to mitigate this emerging issue at national and international level. Furthermore, one health training should include cross-cultural communication skills, team building, and trust development. One Health requires institutions that cultivate expertise in areas such as leadership, advocacy, partnership, knowledge translation, evidence based decision making and capacity building. This issue also needs dire attention of all the stakeholders i.e. legislative, administration, policy makers, corporate sector, professionals especially related to medical and biological studies and above all the general public itself. These collaborative endeavours will be helpful to mitigate the issue of food safety and eventually to achieve the one health.

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Conflict of interest

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