Original Research

Application of High-Performance Thin-Layer Chromatography and Bioautography Techniques for Determination of Antibiotic Residues in Mastitic Cow Milk Following Treatment

Priyanka Das, Kautuk Kumar Sardar*, Gyanaranjan Sahoo and Subas Chandra Parija

Department of Pharmacology and Toxicology, College of Veterinary Science and A.H., Orissa University of Agriculture and Technology, Bhubaneswar - 751 003, Odisha, INDIA

***Corresponding author:** kksardar@gmail.com

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Abstract

A method to identify and quantify multiple antibiotic residues like ceftriaxone and enrofloxacin in mastitic cow's milk by high-performance thin-layer chromatography coupled with bioautography was studied. The antibiotic residues were extracted from antibiotic treated cow's milk suffering from mastitis with the help of acetonitrile by eliminating fat using petroleum ether and finally isolated with dichloromethane. The chromatogram of peaks of antibiotic residue confirmed the presence of antibiotics in the milk samples with presence of 0.108±0.011mg/ml (ceftriaxone) and 0.173±0.015mg/ml (enrofloxacin) in the milk sample, collected 10 days post-administration of drug with initial dose of 15g of ceftriaxone and 9g of enrofloxacin respectively. In the present study, the presence of antibiotic residue beyond 10 days is contradictory from the withdrawal period given by the pharmaceutical company for 5 days. Bioautography confirmed the presence of active antibiotic residue in milk sample-1, 2, 3, 4, 5 and 6 with zone of inhibition of 6.5±0.407mm, 6.0±0.495mm, 5.0±0.666mm, 5.2±0.4mm, 5.1±0.432mm and 6.0±0.401mm, respectively. The test microorganism used for bioautography was Streptococcus spp.

Key words: Antibiotic Residue, Bioautography, Ceftriaxone, Enrofloxacin, HPTLC, Milk

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Introduction

Milk is one of the most nutritionally complete foods available and its consumption has been promoted worldwide. Milk containing proteins, saturated fat, calcium, vitamins, etc. shares a significant proportion of the daily essential nutrients required for human growth and development of all age groups (Connie, 2010). Milk recommended for children and elderly women should be free from antibiotic residues

(Goulding *et al*., 2004). Mastitis is the most prevalent disease in dairy cows making milk unfit for human consumption vis a vis serves a source of spread of diseases such as streptococcal intoxication, streptococcal sore throat, colibacillosis, tuberculosis and brucellosis. Mastitis inflicts heavy economic loss to the dairy farmers in India mainly due to loss in milk production and quality (Radostitis *et al*., 2007; Das *et al*., 2016). Owing to involvement of heavy financial implication and the inescapable existence of latent infection, mastitis is obviously a principal factor that limits dairy production and warrants immediate antibiotic therapy. Antibiotics are employed widely in veterinary practice for both prophylaxis and treatment of mastitis in dairy cows and are added directly to milk to prolong their freshness.

Ceftriaxone is used widely in the treatment of mastitis in cows as well as both human and veterinary diseases because of their broad spectrum of antibacterial activity and good pharmacokinetic properties (Sar *et al*., 2014). Enrofloxacin having bactericidal nature dominates the veterinary practice because of its high therapeutic value (Lopez *et al*., 2015). Ceftriaxone and enrofloxacin, two widely used veterinary antibiotics, may be present as residues in milk following treatment and hence there is an urgent need for their detection and determination. The presence of drug residues in milk will have public health implications, and hence knowledge about the penetration of ceftriaxone from blood to milk is worth consideration (Goudah *et al*., 2006).

In many countries, Government authorities have established monitoring programs for antibiotic determination in food (Ministry of Agriculture, HMSO, London, 1992). The monitoring of the veterinary drug residues is an important component of the food safety control in various raw materials and foods of animal origin, and maximum residue limits (MRL) of veterinary drugs recruited with the food animals have been set to ensure the safety of foods of animal origin for consumers (Commission Regulation 37/2010; Elizabeta *et al*., 2011; Navratilova *et al*., 2011; Langiano *et al*., 2012). Several techniques like thin-layer chromatography (TLC), gas–liquid chromatography (GLC), radioimmunoassay, electrophoresis, high-performance liquid chromatography (HPLC), high performance thin layer liquid chromatography (HPTLC) as well as microbiological and immunological assays are heavily relied to determine antibiotic residues in milk. The coupling of TLC with microbiological detection (bioautography), a simple, cheap and quite sensitive method, has been used for the identification and quantification of several antibiotics (Petz *et al*., 1987; Choma and Grzelak, 2011).

To the best of our knowledge, no currently available studies have identified the presence of ceftriaxone and enrofloxacin residue in milk collected from mastitic cows following treatment at dairy farmers' door step. Therefore, the present study was undertaken to determine the antibiotic residues in mastitic cow milk following treatment.

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Materials and Methods

Standard Antibiotic Solutions and Solvent System

Stock solutions: Ceftriaxone (M/s Intas Pharmaceuticals Ltd., Gujarat, India) and enrofloxacin (M/s Intervet India Pvt. Ltd., Pune, India) were dissolved individually in methanol at a concentration of 10 mg/ml and stored at 4 °C. The standards were prepared from the antibiotics used in treatment of mastitis in cows in field conditions. Each stock solution was diluted with 1ml of methanol to make a final concentration of 1mg/ml. The developing solvent used for HPTLC was a mixture of dichloromethane:acetone: methanol:glycerin (64:20:15:1 v/v) (Ramirez *et al*., 2003).

Selection of Animal and Collection of Milk Sample

The study was undertaken in crossbred Jersey dairy cows (n=67) suffering from mastitis which were treated with ceftriaxone and enrofloxacin by the field veterinarians. Of them, 30 cows treated with ceftriaxone and enrofloxacin maintained by the livestock farmers were selected in the present study, and were divided into six groups. Milk samples (20 ml) were collected from each antibiotic treated mastitic cow that got cured completely after treatment. Group-1 were treated with ceftriaxone @ 3g/day for 5 days, and milk samples were collected after 7days post-treatment. Group-2 cows were administered with enrofloxacin @ 1.5 g /day for 6 days and milk samples were collected 10 days after treatment. Similarly, the Group-3 cows were treated with ceftriaxone @ 2g/day for 5days and milk samples were collected after 10 days of treatment. The Group-4 cows were injected with enrofloxacin @ 1.5 g/day for 5 days and milk samples were collected after 10 days of treatment. Likewise, Group-5 and Group-6 cows were treated with ceftriaxone @ 2g/day for 6 days and 3g/day for 4 days, respectively, and milk samples were collected after 10 days post-treatment.

Extraction of Sample

The whole procedure was conducted in the Central Instrumentation facility, Orissa University of Agriculture and Technology, Bhubaneswar. The Malisch multiresidue method for the determination of residues of chemotherapeutics was used to extract the analytes (Malisch, 1986). Milk sample (20 ml) from mastitic cows were extracted by thoroughly mixing in a commercial blender at a high speed with acetonitrile (40ml) for 1 min. The liquid phase was decanted into 250 ml separating funnel. Petroleum ether (20ml) was added and shaken for 1 min, after which the upper phase was discarded. Then sodium chloride (2g) was poured carefully into the separating funnel and shaken gently to dissolve as much as possible. After that 30ml dichloromethane was added to it and shaken again for 1 min, then the lower phase was drained into a round bottom flask and evaporated to dryness at 40 ^{o}C in a rotary vacuum evaporator. Then the residue was reconstituted with 1ml methanol and put in the eppendorf tube to be used for spotting on HPTLC plate.

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Method for HPTLC

Chromatography was done on a silica gel HPTLC plate (10 × 10 cm, silica gel $60F_{254}$, 200 µm thickness; M/s E Merck, Germany). Each sample group (5µl) was applied 10 mm above the base of the plate using application device 'LINOMAT- 5', (M/s CAMAG, Switzerland with all the accessories) fitted with a syringe of 100 µl capacity using nitrogen gas at 6 psi followed by air drying of the HPTLC plate at room temperature. The plates were then developed in a twin trough chamber using the mobile phase dichloromethane:acetone:methanol:glycerin (64:20:15:1 v/v). The plates were developed in a pre-equilibrated closed chamber till the solvent reached to 90% height of the plates. The plates were removed and the solvent fronts were marked and dried on a plate dryer. The developed plates were visualized at 254 nm and 366 nm under a visualizer and photographed by the documentation system. Then the plates were scanned under CAMAG TLC SCANNER-3 at 254 nm and 366 nm. The chromatographic conditions were optimized previously to achieve the best resolution and peak shape. The concentration in the unknown sample peak corresponding to the standard peak was calculated by comparing the peak area (AU) of the peak to that of the peak of the standard and the respective concentration of the standard solution, by using the formula: AU (sample peak) / AU standard peak x concentration of standard.

Bioautography

Milk samples (n=30) collected from mastitic cows in and around Bhubaneswar including other areas of Odisha State were cultured on Edward medium agar for isolation of the organism. The Gram positive bacteria were isolated and identified up to genus level as streptococci based on the morphology, cultural and biochemical reactions employing CAMP test (Bhagat *et al*., 2015). In bioautography, Molten Mueller–Hinton agar was taken in a petridish and spread all over the petridish and left for drying. The broth containing culture of *Streptococcus agalactiae* was applied on the surface of the petridish. The HPTLC plates were cut into uniform pieces containing antibiotic residue and put on the petridish in inverted manner, and the petridish were then incubated for 48 h. Inhibition zone diameters were measured with Vernier calipers and compared with antibiotic standards inhibition zones (Ramirez *et al*., 2003).

Statistical Analysis

All the experimental results were expressed as mean ± standard error (SE) in replicates (n=5).

Result and Discussion

Detection of Antibiotic Residue

The antibiotic residue in milk sample collected from six groups were shown in Table 1. The standard-1 showed a single peak that started at 0.87 R_f and ended at 0.94 R_f having area of 7092.8 AU (arbitrary units)

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containing ceftriaxone. The standard -2 gave six peaks of which one peak was from 0.43 R_f to 0.77 R_f of area 108812.6 AU showing presence of enrofloxacin. It also showed another peak which corresponded to peak of standard -1 (ceftriaxone) with start position 0.82 R_f and end position 0.99 R_f having area of 19152.1 AU.

Table 1: Rf value, peak position and peak area of scanned HPTLC plate

Track	Peak	Max. Position	Max. Height	Max. %	Area	Area %	Assigned Substance
1	1	0.03R_f	11.7AU	7.03%	115.2AU	1.60%	Unknown
1	2	0.94R_f	1545 AU	92.97%	7098.8 AU	98.40%	Ceftriaxone
2	1	0.02 R_f	42.7 AU	3.50%	290.4 AU	0.22%	Unknown
2	2	0.05 R_f	62.3 AU	5.12%	1242.4 AU	0.93%	Unknown
2	3	0.10 R_f	19.5 AU	1.60%	329.0 AU	0.25%	Unknown
2	4	0.29 R_f	54.6 AU	4.48%	3275.7 AU	2.46%	Unknown
2	5	0.68 R_f	708.1 AU	58.11%	108812.6 AU	81.75%	Enrofloxacin
2	6	0.93 R_f	331.4 AU	27.19%	19152.1 AU	14.39%	Unknown
3	1	0.01 R_f	63.3 AU	13.72%	424.8 AU	2.25%	Unknown
3	2	0.14 R_f	20.6 AU	4.47%	626.5 AU	3.32%	Unknown
3	3	0.73 R_f	300.5 AU	65.20%	14152.1 AU	75.00%	Unknown
3	4	0.94 R_f	76.5 AU	16.61%	3666.6 AU	19.43%	Unknown
4	1	0.02 R_f	11.8 AU	7.50%	69.5 AU	1.13%	Unknown
4	2	0.14 R_f	18.8 AU	11.95%	708.1 AU	11.51%	Unknown
4	3	0.61 R_f	21.6 AU	13.72%	491.6 AU	7.99%	Unknown
4	4	0.69 R_f	53.5 AU	33.97%	2368.0 AU	38.49%	Unknown
4	5	0.95 R_f	51.7 AU	32.86%	2515.8 AU	40.89%	Unknown
5	1	0.02 R_f	12.9 AU	11.87%	117.1 AU	2.90%	Unknown
5	2	0.14 R_f	20.9 AU	19.33%	535.9 AU	13.27%	Unknown
5	3	0.64 R_f	19.4 AU	17.93%	711.1 AU	17.61%	Unknown
5	4	0.95 R_f	55.1 AU	50.86%	2674.2 AU	66.22%	Unknown
6	1	0.01 R_f	120.4 AU	62.73%	901.7 AU	22.41%	Unknown
6	2	0.14 R_f	13.2 AU	6.89%	275.8 AU	6.85%	Unknown
6	3	0.94 R_f	58.3 AU	30.38%	2847.0 AU	70.74%	Unknown
7	1	0.01 R_f	82.3 AU	48.45%	658.3 AU	14.78%	Unknown
7	2	0.14 R_f	15.4 AU	9.04%	363.9 AU	8.17%	Unknown
7	3	0.94 R_f	72.3 AU	42.52%	3432.3 AU	77.05%	Unknown
8	1	0.14 R_f	10.4 AU	4.98%	99.8 AU	1.26%	Unknown
8	2	0.65 R_f	33.7 AU	16.22%	1231.7 AU	15.50%	Unknown
8	3	0.66 R_f	36.1 AU	17.38%	1116.1 AU	14.05%	Unknown
8	4	0.87 R_f	30.7 AU	14.79%	1079.0 AU	13.58%	Unknown
8	5	0.94 R_f	96.9 AU	46.63%	4419.8 AU	55.62%	Unknown

Milk sample (Group -1) showed the area 14152.1 AU starting from 0.61 R_f to 0.77 R_f containing ceftriaxone. Milk sample (Group-2) demonstrated peak starting from 0.90 R_f to 1.00 R_f with area 2515.8 AU containing enrofloxacin. It also gave another peak corresponding to ceftriaxone starting from 0.64 R_f to 0.72 R_f with an area of 2368.0 AU. Similarly, milk sample (Group-3) and (Group-6) showed peak corresponding to both ceftriaxone and enrofloxacin. Milk sample (Group-4) contained only enrofloxacin residue with a peak area of 2847.0 AU that started from 0.89 R_f to 0.99 R_f.

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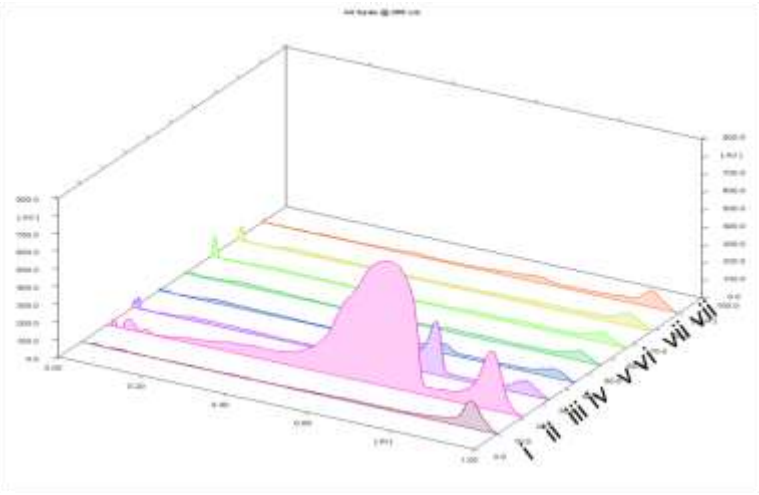

Fig. 1: Chromatogram (254 nm) showing antibiotic residue in the milk of mastitic cows having eight peaks i) Ceftriaxone, ii) Enrofloxacin, iii) Group -1, iv) Group -2, v) Group -3, vi) Group -4, vii) Group -5 and viii) Group -6

The chromatogram peaks of antibiotic residues at 254 nm were shown in Fig.1 and the photographs of the scanned HPTLC plate at 366 nm and 254 nm (Fig. 2a and 2b), confirming the presence of antibiotics in the milk samples. The standard-1 contained the ceftriaxone (1mg/ml) while standard-2 contained both enrofloxacin (1mg/ml) and ceftriaxone (0.34 mg/ml) (Table 2).

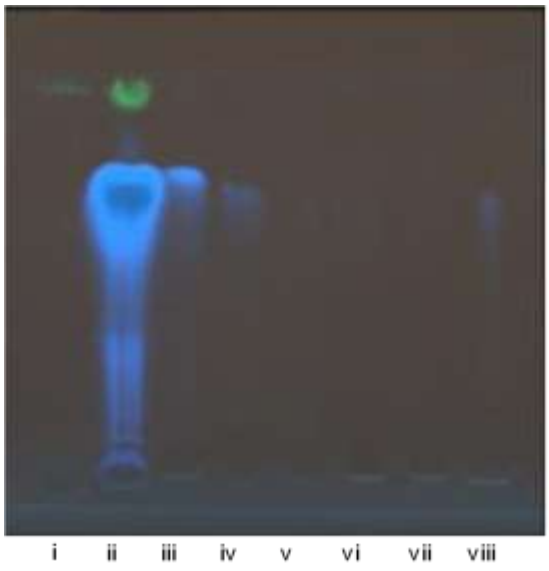

Fig. 2: (a) Photograph of HPTLC plate at 366 nm

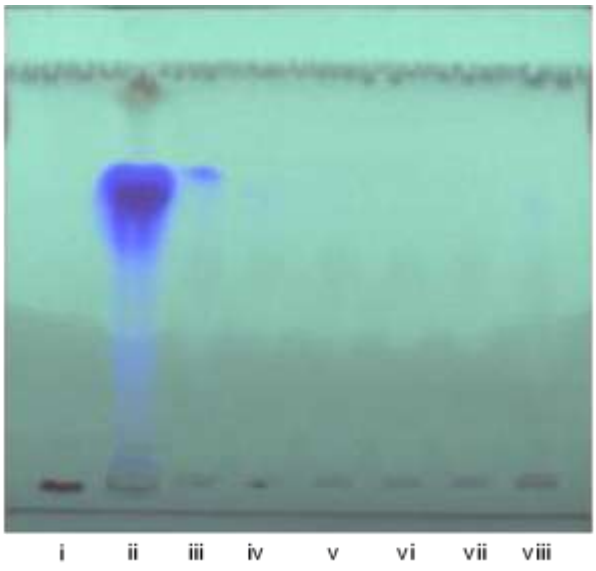

Fig. 2(b): Photographed at 254 nm showing eight different lanes i) Ceftriaxone, ii) Enrofloxacin, iii) Group -1, iv) Group -2, v) Group -3, vi) Group -4, vii) Group -5 and viii) Group -6

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Table 2: Determination of antibiotic residue in milk sample (n=30) using HPTLC

Sample	Total dose of Administration	Name of the Antibiotic Given	Total Antibiotic Given	Amount of Ceftriaxone (mg/ml)	Amount of Enrofloxacin (mg/ml)
Group No.(n=30)	(dose x days)				
S_1				1mg/ml	_
S_2				0.34mg/ml	1mg/ml
1	3gx5	Ceftriaxone	15g	0.418±0.023mg/1ml	_
2	15mlx6	Enrofloxacin	9g	0.108±0.011mg/1ml	0.173±0.015mg/ml
3	2gx5	Ceftriaxone	10g	0.220±0.009mg/1ml	0.039±0.012mg/ml
4	15mlx5	Enrofloxacin	7.5g		0.156±0.015mg/ml
5	2gx6	Ceftriaxone	12g	0.282±0.012 mg/1ml	_
6	3gx4	Ceftriaxone	12g	0.370±0.016mg/1ml	0.061±0.018mg/ml

As we took enrofloxacin injected to the animals as standards, the chromatogram showed two peaks containing both enrofloxacin of 1mg/ml and trace amount of ceftrixaone of 0.34 mg/ml. Milk sample (Group-1) contained only ceftriaxone (0.418± 0.023 mg/ml) with total administered dose (15g) of injection of ceftriaxone intramuscularly and the milk sample was collected after 7days of treatment. There was presence of 0.108±0.011 mg/ml (ceftriaxone) and 0.173±0.015mg/ml (enrofloxacin) with total dose (9g) of enrofloxacin (Group-2), and the milk sample was collected after 10 days of post-administration. Similarly, the cows treated with ceftriaxone (10g) in Group-3 displayed ceftriaxone (0.220±0.009 mg/ml) and enrofloxacin (0.039±0.012 mg/ml) after 10 days of drug administration. The Group-4 cows administered with enrofloxacin (7.5g) and the Group-5 cows injected with ceftriaxone (12g) contained enrofloxacin and ceftriaxone with concentration of 0.156±0.015 mg/ml and 0.282±0.012 mg/ml, respectively, 10 days post-treatment (Table 2). The Group-6 cows treated with ceftriaxone (12g) recorded 0.370±0.016 mg/ml (ceftriaxone) and 0.061±0.018 mg/ml (enrofloxacin) after 10 days of post-administration (Table 2).

The experiment showed that milk sample (Group-1) and sample (Group -5) contained ceftriaxone residue of 0.418±0.023 mg/ml and 0.282±0.012 mg/ml, collected after 7 days and 10 days of post-treatment, respectively. But in case of standard -2, both ceftriaxone and enrofloxacin were detected. The milk sample of Group - 2, 3 and 6 contained both the antibiotic residues and the milk sample of Group-4 had only enrofloxacin. In our study, enrofloxacin was estimated to be 0.173±0.015 mg/ml, 0.039±0.012mg/ml, 0.156±0.015 mg/ml and 0.061±0.018mg/ml in milk sample of Group-2, -3, -4 and -6, respectively, after 10 days of treatment which showed peak value of standards and samples (Fig. 1). Presence of veterinary antibiotic residue on chloramphenicol, sulfonamides, tetracyclines, gentamicin, streptomycin, dihydrostreptomycin, flumequine and enrofloxacin in milk was studied by Bilandzic *et al.* (2011) who detected 4.11 µg/L of enrofloxacin in three months duration contrast to the present study. However,

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consistent to our finding, European Commission regulation (EU) No. 37/2010 has also reported that the MRL value of ceftriaxone (Cephalosporin) and enrofloxacin (Fluroquinolone) in milk is 100 µg/kg each and recommended use of thin-layer chromatography-direct bioautography for the screening of ciprofloxacin and enrofloxacin at the maximum residue level stipulated for milk (Commission Regulation (EU) No. 37/2010; Navratilova *et al*., 2011).

In the present study, in Group -1, the ceftriaxone residual amount was found to be 0.418±0.023 mg/ml after 7days of treatment, whereas, Johal and Srivastava (1998) reported ceftriaxone concentration in plasma measured at 8h in crossbred calves to be 0.32 ± 0.03 mg/ml with initial dose of 10mg/kg.. Following single intramuscular dosing, Sar *et al.* (2013) studied that ceftriaxone exhibited absorption-reabsorption pattern in plasma. Kumar *et al.* (2010) detected MIC_{90} of 0.5 $\mu g.ml^{-1}$ for ceftriaxone in milk 36 h post-administration. Ceftizoxime was also available from 72-360 h post-dosing in milk in presence of fibrosin following intramammary administration of ceftriaxone suggesting that the polyherbal drug played a key role in the penetration of ceftriaxone from milk to systemic circulation (Sar *et al*., 2011, 2014). Similar study was done by Baynes *et al.* (2016) confirmed that the U.S. milk withdrawal time for cattle treated with tetracycline was 96 h. Study done by Buket *et al.* (2013), revealed that the mean levels of quinolones were found to be 30.81 ± 0.45 µg/kg and 6.64 ± 1.11 µg/kg in chicken and beef samples, respectively, by ELISA method where the samples were collected randomly from local market within one month. According to study of Do *et al.* (2016), the sulfamethazine residues were figured out at levels ranging from 11-1600 mg/kg in pork meat by LC-MS/MS method where the samples were collected from local market at a random basis. In the study of Kawalek *et al.* (2016), the highest concentrations of florfenicol was 1.6 ± 2.2 µg /ml of milk at 22 h of administration of drug with initial dose of 2.5 mg /kg body weight following intramammary infusion (Shanoy *et al*., 2016). Liquid chromatography tandem-mass spectrometry demonstrated that florfenicol residues concentration ranged from 0.4 - 0.6µg/g in liver of white-tailed deer after 10days of drug administration with initial dose of 20 mg/kg body weight, intramuscularly (Anderson *et al*., 2016). For qualitative and quantitative determination of florfenicol in white-tailed deer tissues, the study was done by utilising solid phase extraction and liquid chromatographic separation followed by mass-spectroscopy product and parent ion pattern.

Detection of sulfathiazole in milk was 30 ng/ml using magnetic solid phase extraction-HPLC-UV method (Osboo *et al*., 2015). Chowdhury *et al.* (2015) reported that the average concentrations of amoxicillin residue in local milk and local egg were 9.84 µg/ml and 10.46 µg/g, respectively, where the samples were collected randomly from local household farms at Raozan Upazila, Bangladesh, whereas, amoxicillin residue was found to be 56.16 µg/ml and 48.82 µg/g for commercial milk and commercial egg, respectively, collected from commercial farms in the region of Chittagong Metropolitan area in Bangladesh. The study established that milk collected from commercial dairy farm and eggs from commercial layer farms had

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higher concentration of antibiotic residues than milk and egg from local farms which might be attributed to indiscriminate use and improper monitoring system for antibiotic withdrawal period of specific antibiotics in the commercial dairy and layer farms (Chowdhury *et al*., 2015). Drug residues in milk have a potential hazard for the consumer and may cause several adverse reactions, interfere the intestinal flora and develop resistant populations of bacteria, thereby rendering antibiotic treatment ineffective (Dewdney *et al*., 1991). An efficient control of the residues in milk is very important to ensure the safety of milk and milk products (Navratilova *et al*., 2011).

In the present study, the amount of antibiotic residue present in milk sample 10 days post administration revealed that enrofloxacin was better than ceftriaxone from public health point of view as lower residue was present in milk compared to ceftriaxone and its half-life is also less than ceftriaxone.

Antibiotic Sensitivity Test for Antibiotic Residue in HPTLC Plate (Bioautography)

Antibiotic sensitivity test of the HPTLC plate containing antibiotic residue showed the zone of inhibition (Table 3 and Fig. 3).

Table 3: Antibiotic sensitivity test for antibiotic residue in HPTLC plate (Bioautography)

Sample Group No. (n=30)	Zone of Inhibition (in mm)	Assigned Substance
S_1	8	Ceftriaxone
S_2	7.1	Enrofloxacin
1	6.5±0.407	Sample gr-1
2	6.0±0.495	Sample gr-2
3	5.0±0.666	Sample gr-3
4	5.2±0.405	Sample gr-4
5	5.1±0.432	Sample gr-5
6	6.0±0.401	Sample gr-6

Fig. 3: Antibiogram showing presence of active antibiotic residue in the HPTLC plate

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Ceftriaxone and enrofloxacin demonstrated the zone of inhibition of 8.0 mm and 7.1 mm, respectively. The detected antibiotic residue present in milk sample (Group - 1) elicited the zone of inhibition of 6.5±0.407 mm. Similarly, other milk sample (Group -2 to Group - 6) showed the zone of inhibition of 6.0±0.495 mm, 5.0±0.666 mm, 5.2±0.4 mm, 5.1±0.432 mm and 6.0±0.401mm, respectively. Choma (2006) screened enrofloxacin and ciprofloxacin residues in milk by HPLC followed by TLC with direct bioautography. Moreover, in TLC-direct bioautography technique, semi-quantitative determination of flumequine and cefacetril in milk was also determined by TLC-DB which was further compared with quantitative HPLC analysis (Piech *et al*., 2016). The zone of inhibition showed by enrofloxacin was less than that of ceftriaxone showing less residual amount of antibiotic in milk (Table 3).

Ceftriaxone and erofloxacin as standards showed the zone of inhibition of 8.0 mm and 7.1 mm, respectively. Enrofloxacin showed the zone of inhibition of 5.2±0.4 mm in Group- 4 and ceftriaxone with 6.5±0.407 mm zone of inhibition in Group-1 whereas Ramirez *et al.* (2003) recorded zone of inhibition of 8.5 mm, 6.5 mm and 5.5 mm for ampicillin, chloramphenicol and dicloxacillin, respectively, where the milk was fortified with each antibiotic working standard solution to a level of 0.1 mg/ ml. The maximum diameter of zone of inhibition observed from muscle of chicken containing chloramphenicol residue was 3.8 ± 0.4 mm for *Staphylococcus aureus* (Tajik *et al*., 2010), collected arbitrarily from five different poultry meat center in northwest of Iran. According to study of Patil *et al.* (2013), the zone of inhibition using 2.0 mg/ml phenyl tetrazolium chloride against *Bacillus cereus* and *Sclerotium rolfsii* were 30mm and 20mm, respectively, in biautography method. The maximum zone of inhibition of *Staphylococcus aureus* was recorded by using dimethyl sulphoxide extracts of green tea variety, qimen was 10.00±0.0 mm by bioautography method which represents the antibacterial property of qimen (Bashir *et al*., 2014).

Conclusion

One goal of total quality management is to prevent the occurrence of antibiotic residue in raw milk shipped from the dairy farmers to consumers. The potential of HPTLC and bioautography as a rapid analytical tool for the detection and determination of antibiotic residue in milk were demonstrated. The presence of ceftriaxone and enrofloxacin residue in milk sample collected from mastitic cows was detected through HPTLC that brought to a close confirmation about the milk withdrawal period to be maintained by the consumers from public health concern. The finding from this study will serve as a basis in developing a database related to presence of antibiotic residues in milk for wellbeing and safe human consumption.

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