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Assessment of oxytetracycline residue by microbial assay and high performance thin layer chromatography in milk

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Abstract
Aim: This study aims to detect oxytetracycline residue in milk by a simple microbial screening and chromatographic method.
Methodology: A total of 200 milk samples were collected from organized dairy farms in and around Chennai. Milk samples were screened for the presence of oxytetracycline antibiotic residues by microbial assay using *Bacillus subtilis* MTCC 121^T and antibiotic residue positive samples and were confirmed by High Performance Thin Layer Chromatography.
Results: The results revealed that 19 milk samples were positive for oxytetracycline residue in milk by microbial assay and was confirmed by High Performance thin layer chromatography with average concentration of 139.25 µg/kg and 78.59 µg/kg in cow and buffalo milk respectively.
Conclusion: The study infers that the microbial screening method is a simple and economical method that can be used to detect antibiotic residues in foods of animal origin.

Keywords: Antibiotic residues, milk, microbial screening, oxytetracycline (OTC), (HPTLC)

1. Introduction
Antibiotics are widely used for the treatment of milch cows and buffaloes. The residues of these drugs may remain in milk and dairy products which can be a potential threat to human health. There is a risk of development of resistant strains of human bacteria due to the exposure of antibiotics in low levels which in turn may result in public health problem (Trombete *et al.* 2014) [1].
Antimicrobials were first used in veterinary medicine for the treatment of mastitis in dairy cows. The most commonly used antimicrobials in food animals are beta-lactams (e.g., penicillin and cephalosporin), tetracycline (e.g., oxytetracycline, tetracycline, and chlortetracycline), aminoglycosides (e.g., streptomycin, neomycin and gentamicin), macrolides (e.g. erythromycin) and sulfonamides (e.g., sulfamethazine). In addition to their therapeutic value, the discovery of the ability of antibiotics to enhance growth and feed efficiency of food animals in 1950 there was widespread use of antibiotics as feed supplements. The presence of antibiotic residues (AR) in milk was a problem for the dairy industry as it inhibited the starter culture (Mitchell *et al*. 1997) [2].
The overuse of antibiotics in agricultural production, as well as the presence of residues in the food chain, has expressed many human and animal concerns. Penicillin induced allergic reactions in sensitized individuals, chloramphenicol caused toxicity such as aplasia of the bone marrow, effects on the human gut microbial populations, the emergence of resistant bacteria within animals and the transfer of antibiotic resistance genes to human pathogens, and nitrofurans have been found to be animal carcinogens and mutagens in genotoxic tests (Mitchell *et al*., 1997) [2].
Codex Alimentarius Commission set guidelines by the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) based on Joint WHO/FAO Expert Committee on Food Additives (JECFA) for the determination of Maximum residue limits (Codex Alimentarius Commission, 2012). The presence of violative levels of residues in foods are illegal and subject to financial penalties in many countries. Thus, there is a need for antibiotic residue detection methods to reduce residues in foods of animal origin (Vishnuraj *et al*. 2016) [3].

The antibiotic residue detection in milk and food is possible by chemical methods, microbiological methods and immunological assays and they can provide a qualitative or quantitative data about residue level. Thus the regulatory bodies should be formed to control the antimicrobial residue level in bulk milk before consumption (kebede *et al*. 2014) [4].
Out of the numerous antibiotic detection methods, the microbiological assay is the first step in screening samples for the presence of antibiotic residues (Hakimzadegan *et al*. 2014) [5].
This study is performed to detect the existence of antimicrobial residues in milk by microbial assay and chromatographic method.

2. Materials and Methods

2. a. Standard

Certified Reference Material (CRM) of the antibiotic standard Oxytetracycline was obtained from M/S Neospark with a purity standard of 99% and with certificate of analysis. The standards were prepared by diluting in methanol in amber colored volumetric flasks, labeled and kept in stock. Working standards were suitably diluted and can be used for HPTLC analysis. Pre-coated aluminum oxide plates of M/S, E-Merk (India) Ltd were used. Plates measuring 10×8 (cm) plates were used for spotting three samples against authenticated reference standards.

2. b. Chemicals

The chemicals used for the extraction of Oxytetracycline from milk samples for HPTLC analysis of extracts were of analytical grade and purchased from Merck and Sigma-Aldrich.

3. c. Sample collection

A total of 200 milk samples which comprises 100 cow and 100 buffalo milk, were collected from organized dairy farms in and around Chennai, Tamil Nadu, India. The milk samples were kept in the refrigerator until further use.

2. d. Preparation of culture medium

The following culture media were used: nutrient agar and nutrient broth obtained from HIMEDIA. Nutrient broth (1.3 g) was weighed in a conical flask and 100 ml of distilled water was added, as per the manufacturer's instructions. It was then sterilized in an autoclave at a pressure of 15 mm Hg and a temperature of 121°C for 15 minutes, after which it was cooled to about 50°C (Fig.1) Then Bromocresol Purple indicator (0.002 g/l) was added to the autoclaved nutrient broth medium (Tharanya *et al*. 2018) [6] (Fig.2).

Fig 1: Nutrient broth

Fig 2: Nutrient broth with dye

2. e. Preparation of bacterial strain

The bacterial strain used was *Bacillus subtilis* (MTCC 121^T), obtained from Microbiological Type Culture Collection (MTCC). The freeze-dried bacterial culture was activated according to the instructions given by MTCC.
Single colony obtained from the Petri plate (Fig.3) was inoculated into 5ml nutrient broth and incubated at 37ºC for 18-24 hours. Broth suspension of test organism was adjusted with sterile physiological saline to a concentration approximately equal to 0.5 McFarland standard equivalent, to 1.5 $\times 10^8$ CFU/ml. All the procedures were done aseptically under biosafety cabinet (Tharanya *et al*. 2018) [6].

Fig 3: Bacterial colony growth of *Bacillus subtilis* (MTCC 121^T) bacteria on petriplate from the activated freeze dried culture obtained from MTCC Chandigarh

2. f. Extraction for oxytetracycline

Extraction was done as per the method described by Kodimalar *et al*. (2018) [11] with some minor modifications. For extraction procedure, acetonitrile, methanol, distilled water and dichloromethane were used.
The milk sample was taken from the deep freezer and kept for 30 minutes until it reached the room temperature. Four gram of each sample was weighed using a weighing balance and the samples were taken in a sample bottle. 6 ml acetonitrile, 2ml methanol and 2ml distilled water was added to each sample and shaken well in an orbital shaker for 30 minutes. Then the samples were centrifuged at 5000 rpm for 15 minutes. The resultant aqueous supernatant was filtered through Whatman filter paper no. 1 and collected separately. The collected aqueous supernatant was subjected to liquid–liquid extraction by adding 32 mL of dichloromethane in a 500-ml separating funnel. The resultant organic extract was passed through a sodium sulfate bed and collected in a beaker and concentrated in a hot plate under the fume hood. Finally, the dried extract was reconstituted with 100 µl of distilled water and subjected for oxytetracycline antimicrobial residue analysis by microbial tube test method.

2. g. Microbiological tube test method

The antimicrobial drug residues in milk samples were screened using microbiological tube test method with a suitable indicator organism for the antibiotic studied. The Indicator organism *Bacillus subtilis* MTCC 121^T is used to detect the Oxytetracycline residue in milk (Kirbis, 2006) [7].

Standardisation of Oxytetracycline by microbial assay

Working standards 100 ppb, 200 ppb, 500 ppb and 1 ppm. Prepared from Stock standard solution by diluting in water as per the MRL levels set by European Union. The limit of detection (LOD) of Oxytetreacycline with the indicator organism *Bacillus subtilis* was standardised at the permissible Maximum Residue Level (MRL) at 100 µg/kg as set by European union by microbial test tube method.

Procedure
Microbiological tube test was performed as per the method used by Tharanya *et al*. (2018) [6].
1800µl of nutrient broth with pH indicator bromocresol purple was taken in a test tube and then 100µl of extracted milk sample and 100µl of test bacterium *Bacillus subtilis* were pipetted into the test tube and mixed thoroughly. The test tubes with positive growth controls containing culture organism in the broth culture and a negative control containing only the broth were also taken. The test tubes were incubated at 37ºC for 18–24hours. The test tubes that remained purple after the incubation was recorded were positive for antimicrobial residues and those that turned yellow or turbid were recorded as negative for antimicrobial residues.

2. h. High-performance thin-layer chromatographic technique standardisation of oxytetracycline by HPTLC
The standard linearity calibration curve for the antibiotic Oxytetracycline obtained with the mobile phase Dichloromethane: Acetonitrile: 5% EDTA mobile phase in the ratio of 13:4:2 at 366 nm wavelength.

Recovery of oxytetracycline from spiked milk sample
To 4 g of milk sample 100 µg kg^{-1} of Oxytetracycline working standard was spiked and were subjected to extraction and quantification.

Analysis of milk samples by HPTLC
Procedure: HPTLC was adopted as per the method used by Sureshkumar *et al*. (2017) [8]. Thin silica gel plate was cut to the size (10 × 8 cm) and marked with a pencil at the upper edge of the plate for the direction of development. The plate was completely washed with methanol and kept in a hot plate for drying. The samples were spotted (spray-on-technique) using an injector by using Linomat-5 sample applicator. The volume used for spotting was approximately 20 µl. The spotted sample plate was kept in a developing chamber for development which contained the developing solvent. The developing solvent used for oxytetracycline was 13:4:2 ratio of Dichloromethane: methanol: 5% EDTA. The spotted samples were developed up to 80mm from the lower edge of the plate. The plates were viewed under UV light. The plates were then scanned using CAMAG HPTLC scanner-3 under 366nm wavelength.

3. Results and Discussion
3. a. Microbial screening
Standardisation of oxytetracycline by microbial assay
The limit of detection (LOD) of Oxytetreacycline with the indicator organism *Bacillus subtilis* was standardised at 100 µg/kg which is the accepted permissible Maximum Residue Level (MRL) set by European Union. (Table.1 and Fig. 4).

Table 1: Standardisation of Oxytetracycline by microbial assay.

Antibiotic	Bacterial strain	LOD Std.(µg/ kg)	MRL milk (µg/ kg)
Oxytetracycline	*Bacillus subtilis*	100	100

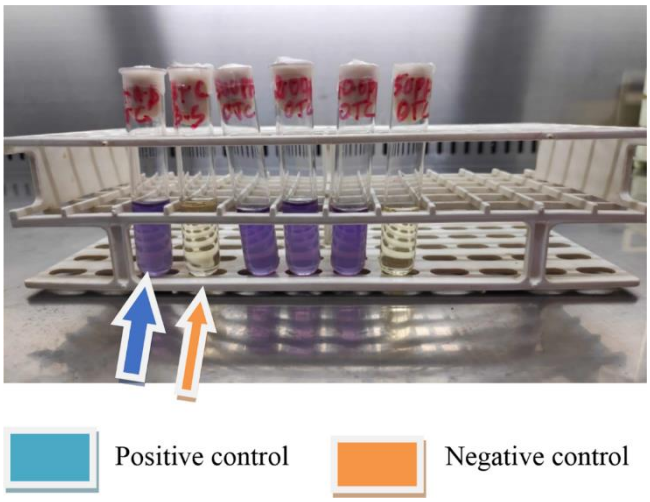


Fig 4: Standardization of Oxytetracycline to assess Limit of Detetion (LOD)

Analysis of milk by microbial screening
Out of the 200 milk samples which is analysed for microbial screening, 13 cow milk and 6 buffalo milk samples were found to be positive for oxytetracycline antibiotic drug residues against the test organism *Bacillus subtilis* (Table 2 and Fig. 5). A positive result was indicated by colour change. The test tubes with purple colour after the incubation were recorded as positive and those that turned yellow or turbid were recorded as negative for antimicrobial residues.

Table 2: Microbial screening of milk using *Bacillus subtilis* for Oxytetracycline.

Milk	Sample Number (n)	Oxytetracycline	
		No. of positive samples	% of positive samples
Cow	100	13	13
Buffalo	100	6	6

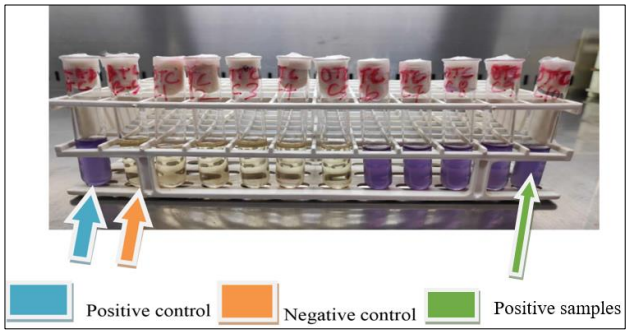


Fig 5: Antimicrobial assay of antibiotic oxytetracycline in milk samples.

Milk is a balanced diet, and is considered one of the world's most complete foods and a rich source of proteins, vitamins and minerals such as calcium, magnesium, phosphorous, potassium and Zinc. The Quality of milk is a subject of worldwide importance because it is a food of high nutritional value and consumed by all populations. Thus milk and milk products intended for human consumption must be safe,

without microbiological, physical or chemical contaminants. (Trombete *et al*. 2014) [1].

Antimicrobials are injected to animals by intramuscular, intravenous and subcutaneous route, orally in the food and water topically on the skin, by intramammary and intrauterine infusions .This may lead to residues in milk, meat and eggs. In U.S. 12% milk supply was adulterated with β-lactams antibiotics prior to 1962.In Britain in 1963, 11% of milk samples tested were found to contain penicillin and in 1988, 75% of North American consumer milk contained detectable levels of tetracycline and sulfamethazin (Mitchell *et al*. 1997) [2].

The use of antimicrobials for food animal production is predicted to increase by 67% in 2030. Hence the use of Antibiotic Growth Promoters (AGP) will lead to antibiotic residues in food. Antibiotic residues cause direct toxicity in humans. Thus there is a need for the antibiotic residue detection methods to reduce residues in foods of animal origin (Vishnuraj *et al*. 2016) [3]. Milk samples from small holder farms in Kenya were screened for antibiotic residues by Delvotest screening test and found the presence of antibiotic residue in milk (Ahlberg *et al*. 2016) [9].

In general monitoring of antimicrobial agent residues, microbial growth inhibition methods, rapid tests, Microbial inhibitor screening methods are employed for detection and also reported that a new rapid test Twin sensor has been developed for detection of more antibiotic groups (Navratilova *et al*. 2008) [10].

3. b. High-performance thin-layer chromatography

Standardisation of oxytetracycline by HPTLC

The standard linearity calibration curve for the antibiotic Oxytetracycline obtained with r = 0.99 (fig.6). The typical representative chromatogram presenting the separation of antibiotic illustrated (fig.7). The Rf value for oxytetracycline by High Performance Thin layer Chromatography is 0.16. The recovery of Oxytetracycline was above 85 % (fig.8).

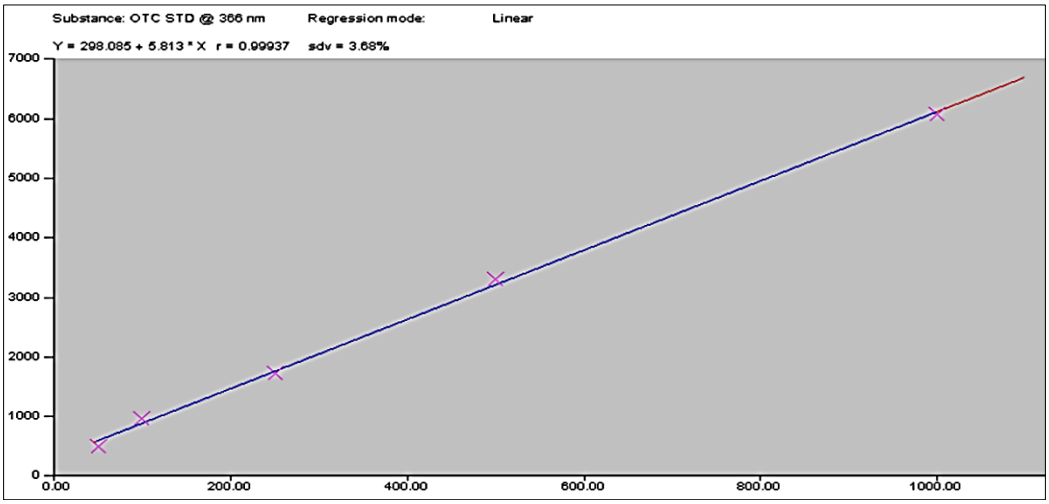


Fig 6: HPTLC Oxytetracycline Standard Linearity calibration curve.

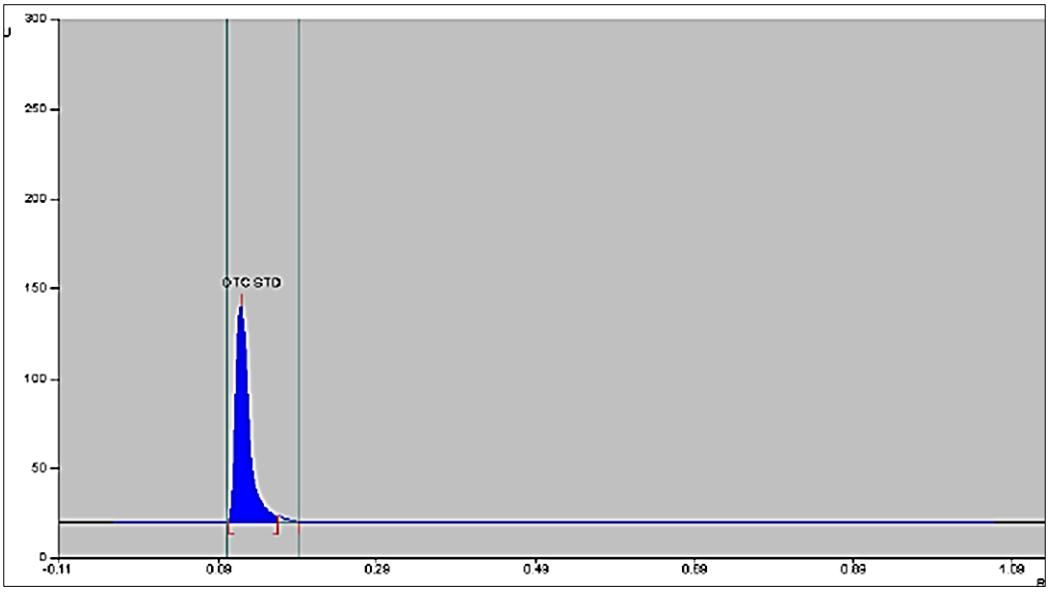


Fig 7: HPTLC chromatogram of Oxytetracycline Standard peak.

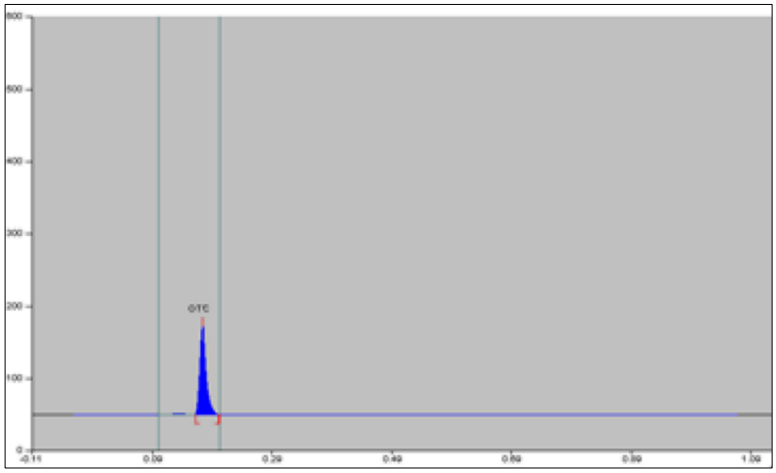


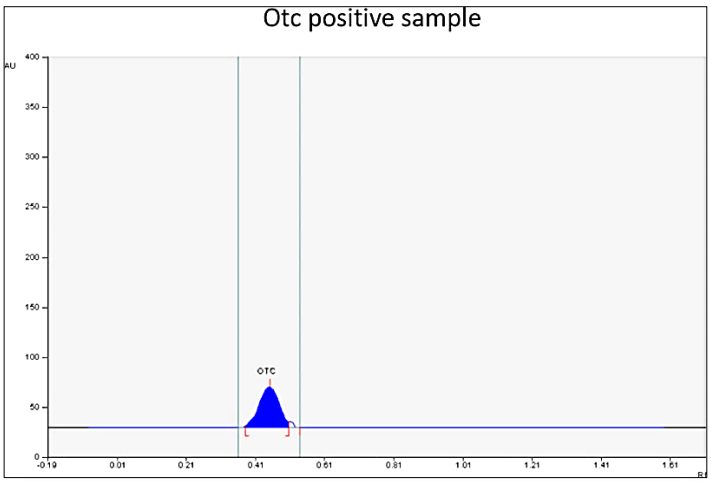
Fig 8: HPTLC chromatogram of Oxytetracycline recovery peak.

Milk samples which showed positive results by microbial screening were subjected to further confirmation and Quantification by High Performance Thin Layer Chromatography (HPTLC). Analysed milk samples were found to be positive for Oxytetracycline in cow and Buffalo milk with a percentage of 13% and 6 % respectively (Fig.10)

The positive 13 cow milk samples for Oxytetracycline residue were in the average concentration of 139.25 µg/kg. The Positive 6 buffalo milk samples for Oxytetracycline residue were in the average concentration of 78.59 µg/kg. The HPTLC Chromatogram with the Oxytetracycline positive milk sample peak is illustrated (Table.3 and Fig.9).

Table 3: .Mean concentration of Oxytetracycline residues in milk by HPTLC.

Milk	**Oxytetracycline**	
	% of Positive Samples	**Mean Concentration** (µg/kg)
Cow	13	139.25 µg/kg
Buffalo	6	78.59 µg/kg



Rf =0.16

Fig 9: HPTLC chromatogram of oxytetracycline positive milk sample

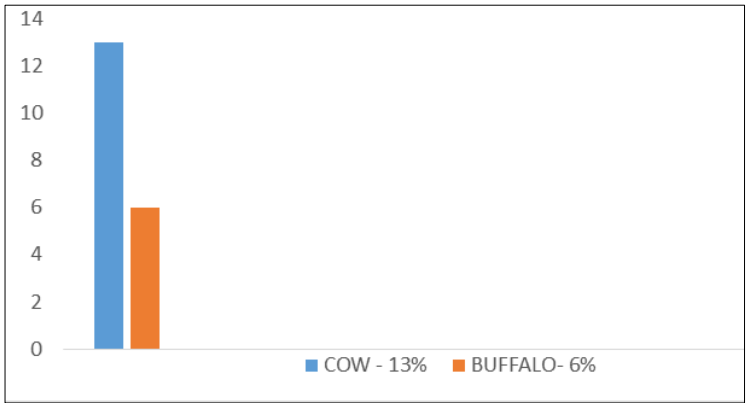


Fig 10: Percentage of oxytetracycline positive samples in milk

Kodimalar *et al*. (2014) [11] reported the residue concentration of Chlortetracycline in chicken egg after a 14 day treatment of chlortetracycline in feed in 12 commercial poultry farms. The 3rd, 5th and 7th day together average concentration of chlortetracycline in feed was quantified by High Performance Thin layer Chromatography as 503.47mg/kg and Chlortetracycline residue level in egg was highest on 7th day with concentration of 206 ug/kg.

In Nazareth dairy farm, a cross sectional study on 400 bulk milk samples between October 2007 and May 2008 was screened for detection of Oxytetracycline antibiotic residues. The study revealed that 48 cow milk samples (12%) were positive for antibiotic residues out of which 40 samples (83.33%) were positive for Oxyteteracycline residue .The mean concentration of the oxytetracycline was 125.25 ug/l by High Performance liquid chromatography which was above the established WTO/FAO/CAC established Maximum residue limit of 100ug/l for oxytetracycline .The study infers that Oxytetracycline has been used imprudently in Nazareth dairy farm (Abebew *et al*. 2014) [12].

Fritz *et al*. (2007) [13] validated Oxytetracycline in Milk samples in Reversed-Phase high-performance liquid Chromatography with photodiode-array detection (HPLC-PAD). The samples were extracted and cleaned up using solid-phase extraction discovery SPE DSC -18 tubes and were separated in Waters Symmetry C18 column with a mobile phase consisting of oxalic acid: acetonitrile: methanol (150:20:20) by volume.

The detection of Oxytetracycline antibiotic residue in milk was validated by High Performance Liquid Chromatography (HPLC) method by using the acetonitrile as mobile phase and reversed phase C18 column as stationary phase at a UV detector wavelength of 325nm (Boultif *et al*. 2014) [14].

The presence of antibiotic residues in animal tissues is due to the insufficient period of time given for the drug to be eliminated from food. Thus, there is a need for following appropriate screening tests to detect residue status in milk. Therefore, for the prevention of antimicrobial residues, the veterinarians and producers should stick to maximum residue limits (MRL) and prescribed withdrawal times of antimicrobial agents (Asredie *et al*. 2015) [15].

4. Conclusion

The overuse of antibiotics in agricultural production as well as the presence of residues in the food chain has expressed many human and animal concerns such as carcinogenic, mutagenic, causing allergic reactions, causing aplasia of the bone marrow, decrease of human gut microbial populations, the emergence of resistant bacteria within animals and the transfer of antibiotic resistance genes to human pathogens. Thus there is a need for the control and monitoring of drug residues in dairy industry by screening assays with a detection level optimised below the unsafe or violative levels (Maximum residue limits) with specified withdrawal period so that there is no health hazard for the consumer.

5. Acknowledgments

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