QUANTITATIVE ANALYSIS OF TETRACYCLINE RESIDUES IN COW AND BUFFALO MILK IN CHHATTISGARH

M.V.Sc. THESIS

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QUANTITATIVE ANALYSIS OF TETRACYCLINE RESIDUES IN COW AND BUFFALO MILK IN CHHATTISGARH

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VITA

The author of this thesis, Praveen Kumar was born on September 25th, 1988 at Surajpur (C.G.). He passed his higher secondary examination in the year 2006 with second division from Sadanand College, Bihar Sharif (Bihar). In July 2006 he started his career in veterinary profession for his B.V. Sc. and A.H. from College Of Veterinary Science, Anjora, Durg (C.G.) and completed it in July 2014 with OGPA of 6.34/10.00. Due to his keen interest in research work towards public health, in the same year he joined Department of Veterinary Public Health and Epidemiology for his master's degree in August 2014. He has completed the requisite course works for M.V.Sc. program.

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CERTIFICATE-I

This is to certify that the thesis entitled **"Quantitative analysis of tetracycline residues in cow and buffalo milk in Chhattisgarh"** submitted in partial fulfillment of the requirements for the degree **"Master of Veterinary Science"** of Chhattisgarh Kamdhenu Vishwavidyalaya, Durg, is a record of bonafide research work carried out by **Praveen kumar**, under my guidance and supervision. The subject of the thesis has been approved by Student's Advisory Committee and the director of Instructions.

No part of the thesis has been submitted for any degree or diploma (certificate awarded etc.) or has been published/published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged by him.

Dr. Sanjay Shakya

Date: **Chairman, Advisory Committee**

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CERTIFICATE-II

This is to certify that the thesis entitled **"Quantitative analysis of tetracycline residues in cow and buffalo milk in Chhattisgarh"** submitted by **Praveen Kumar** to the Chhattisgarh Kamdhenu Vishwavidyala, Durg, in partial fulfillment of the requirements for the degree of **M.V.Sc.** in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science and Animal husbandry, Anjora, Durg, has been approved by the student's Advisory Committee after oral examination in collaboration with the external examiner.

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Anjora, Durg ***Praveen Kumar***

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ABBREVIATIONS

Abbreviation	Full form
PDA	Photo diode array
TCs	Tetracyclines
WHO	World Health Organization
OIE	World Organization for Animal Health
b_w	Body weight
%	Percent
PVDF	Polyvinylidene difluoride
µl	Microliter
µg	Microgram
ml	Mililiter
^0C	Degree celcious
et al.	Et alia (and others)
i.e.	id est (that is)
ADI	Acceptable dietary intake
OTC	Oxytetracycline
TC	Tetracycline
CTC	Chlortetracycline
MRL	Maximum residue limit
EDI	Estimated dietary intake
FAO	Food and Agriculture Organization
HI	Hazard index
HQ	Hazard Quotient
JECFA	Joint Expert Committee on Food and Agriculture

CHAPTER-I

INTRODUCTION

Milk is an important inexpensive dietary source which contains valuable proteins and calcium essential for promoting growth in children and general health of the population. In the modern farming practice, antibiotics are being used in a large scale and are applied in the animal husbandry for different reasons. Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites. Tetracyclines are used routinely in veterinary medicine for prevention and control of diseases. These antibiotics are produced by *Streptomyces spp*, and mode of action is exerted by binding to 30S ribosomal subunits of susceptible bacteria, which in turn inhibits their protein synthesis (Chopra, 1981).

Chlortetracycline and Oxytetracycline, first members of tetracycline group discovered in late 1940s, were products of *Streptomyces aureofaciens* and *S. rimosus*, respectively. Other tetracyclines were discovered later, either as naturally occurring molecules e.g., tetracycline from *S. aureofaciens* or as the product of semisynthetic approaches e.g., methacycline, doxycycline and minocycline (Chopra and Roberts, 2001). The tetracyclines, a large family of antibiotics, were discovered as natural products by Benjamin Minge Duggar in 1945 and first prescribed in 1948. Tetracycline is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system (World Health Organization, 2013). However, the misuse of

these molecules conducts to the presence of their residues in food of animal origin (Wangfuengkanagul *et al*., 2004).

Historically, Tetracyclines are divided into first generation if they are obtained by biosynthesis: tetracycline, chlortetracycline, oxytetracycline, demeclocycline. Second generation if they are derivatives of semisynthesis: doxycycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline and third generation if they are obtained from total synthesis: tigecycline. However, some researchers consider tigecycline a part from tetracyclines drugs as a new family of antibacterial called glycylcycline (Cornell university library). All new compounds with highly bacterial species specific activities (narrow-spectrum) are considered members of fourth generation. Harvard University made available pentacycline antibacterials, a structural modification of Doxycycline with five rings and from other laboratories azatetracycline and alkylaminotetracycline antibacterials. All these compounds are the "logical'' results of modification around the four rings of tetracyclines that historically started with the master work of Golub and McNamara (McNamara, 1986).

It is first-line therapy for Rocky Mountain Spotted Fever (*Rickettsia*), Lyme disease (*B. burgdorferi*), Q fever (*Coxiella*), Psittacosis and Lymphogranuloma venereum (*Chlamydia*), Mycoplasma pneumoniae and to eradicate nasal carriage of meningococci. Tetracycline tablets were used in the plague outbreak in India in 1994. The therapeutic uses are either antibacterial or non-antibacterial and in the literature, the uses fall into five main categories viz: newer and more potent tetracyclines use in antibacterial resistance (Chopra *et al*.,

2001). The nonantibacterial uses of tetracyclines targeted toward inflammation (D'Agostino, 2001) and arthritis (Amin *et al*., 1997), in neurology, antiviral and anticancer and Tet repressor controlled gene switch (Ermak *et al*., 2003). Both laboratory and clinical studies have investigated the anti-Inflammatory properties of tetracyclines, acting as inhibitor of proliferation of lymphocyte (Thong and Ferrante, 1979), suppression of neutrophilic migration (Martin *et al*., 1974), in recent times, TCs have showed to be anti-caking of β-amyloid protein and therefore useful in the treatment of neurodegenerative diseases like Alzheimer's and the Prion Disease (Saracino *et al*., 2008). In bovine, oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) are the major drugs of choice for prevention and treatment of bacterial infections and feed additive. In food producing animals, tetracyclines may be administrated orally through feed or drinking water, parenterally, or by intra-mammary infusion. Due to entero-hepatic circulation, a small amount of administrated dosage may persist in the body for a long period of time after administration (Botsoglou and Dimitrios., 2001).

Today, in addition to the adverse effects that can occur as a result of the use of veterinary drugs, antibiotic resistance is considered to be a major threat to human health. Improper veterinary use of tetracyclines, as well as inadequate knowledge of the necessary withdrawal time, can easily make the tetracyclines or their derivatives appear in the marketed milk. In addition to drug dosage, the levels of those residues depend on the period between administration and collection of the animal products, which is called withdrawal period. Antibiotic residues like other drugs, remain in animals body even after slaughtering, if there has been no enough time to their repel (Wilson *et al*., 2003). The rate of

metabolism of TCs in dairy cows has been estimated 25-75% and a significant percentage of the administrated TCs are excreted in bovine milk. If these antibiotics administered improperly or if the withdrawal time for the treated cows has not been passed, TCs and their degraded products may be present in milk and may cause harmful effects on consumers (Fritz and Zuo., 2007). Antibiotics residues have a negative impact on consumers health and food processing industries. Indeed, the presence of antibiotics residues may harm to the consumers health through direct toxicity (Leroy and Fanir, 2005), Allergic reactions may be produced in susceptible or sensitized individuals (Teale, 2002), and the other hazardous effect is development of resistant strains of bacteria following the prescription of subtherapeutic doses of antimicrobials. It may include transferring of resistance gene of plasmid from nonpathogenic microorganisms to pathogenic microorganisms, which then will not respond to normal drug treatment (Brandsteterova *et al*., 1997), pathologies related to modification of intestinal flora. Tetracyclines have been reported to cause hyporicemia, hypokamemia, proximal and distal renal tubular acidosis in humans (Adetunji, 2008).

For food processing industry, the spoil concern notably manufacturing of fermented products (Form, 2003). In fact, the lactic acid bacteria being sensitive to very low concentrations of antibiotics (Brouillet, 2002), the presence of their residues inhibit partially or totally the growth of these ferments and result in many defects (Robb, 2006) including accidents in cheese manufacturing, yogurt and other fermented milk products (Zinedine *et al*., 2007).

To ensure human food safety, the European Union has set tolerance levels for many drugs in animal products. The maximum residual limits (MRLs) for

tetracyclines in milk were established by European Regulation 2377/ 90 and subsequent modifications. The MRLs for milk were set at 100 µg/kg for all species. Chinese Ministry of Agriculture have established a maximum residue limit of 0.1mg/l for tetracycline, oxytetracycline and chlortetracycline (Zhuang, 1995). The U.S Food and Drug Administration have set the tolerance of 0.3 mg/l for combined residues of TC, OTC and CTC. Methods must be developed for the monitoring of antibiotics residues notably in milk. For that two types of tests are used: microbiological bioassay techniques (screening methods), and physicochemical tests (confirmation methods) (Fergusson *et al*., 2002). Although these methods lack specificity and provide only semiquantitative measurements of residues and sometimes produce false positives. Nevertheless, they continue to be used because of their simplicity and cheapness. Several chromatographic methods have been employed successfully for the monitoring of TCs in milk such as UV-Spectrophotometry, fluorescence, and mass spectrometry. High Performance Liquid Chromatography is recognized as a preferred method for the quantitative analysis of antibiotic residues in milk (Oliveira *et al*., 2007; Reig and Toldra , 2008) since this method can detect very small amounts of these residues, presents an extreme sensitiveness, possesses a great strength of separation and have excellent reproducibility.

In the view of above, the present study was undertaken with the following objectives:

1) Optimization of High Performance Liquid Chromatography protocol for the detection of antibiotic residues in milk.

2) Quantitative analysis of tetracycline residues in cow and buffalo milk samples.

3) Exposure assessment of tetracycline residues in milk.

CHAPTER- II

REVIEW OF LITERATURE

Antibiotics have been widely used in food animal raised in groups and herds to compensate poor production practices. They are used for three main purposes in animals; therapeutic use to treat sick animals, prophylactic use to prevent infection in animals and as growth promoters to improve feed utilization and production (Maraschiello *et al*., 2001; Dipeolu and Alonge, 2002). For their growth promoting properties, they are used as sub-therapeutic levels as animal feed additives (Okerman *et al*., 1997). The growth promoting activities of antibiotics was discovered in 1940s, when it was observed that animal fed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues improve their growth. The mechanism of action of antimicrobial agents as growth promoter is related to interaction with intestinal microflora population (Niebold, 2007).

2.1 History

Penicillin was used in large scale for the first time during World War II. Later lyophilized penicillin was used for the treatment of bovine mastitis (Gustafson and Bowen, 1997).

Stokstad *et al*. (1949) found that, when chlortetracycline was used as feed supplement in poultry, it improved the weight gain of chickens. It also reduced the amount of feed needed to bring broilers to market weight. Other antibiotics also showed similar effects on animals and poultry. The broad-spectrum antibiotics that promoted growth and feed efficiency at low levels shown to control endemic

diseases in large group of animals and poultry. Antibiotics use became popular due to its cost effectiveness.

Swann (1969) highlighted the possible adverse effects of antibiotics on human health by prolonged feeding of sub-therapeutic levels of antibiotics to food animals. Sweden banned the use of antimicrobial growth promoters in food producing animals. As a result of the ban, the use of antibiotics decreased by approximately 55% during the more than a decade. In spite of this antibiotic resistance continued to exist in a proportion of microbial flora (Wierup, 2001).

Miller (1993) reported that Antibiotics constitute the largest proportion of pharmaceutical sales in comparison to any drugs in livestock production. The use of antibiotics has increased to such an extent, that intensive agricultural practices will be impossible to sustain without it (Booth, 1988; Mitchell and Yee, 1995). Veterinarians prescribed antibiotics for treatment of mastitis in lactating dairy cows. It was concluded that penicillins, tetracyclines, sulphonamides and aminoglycosides were most frequently used in lactating animals, which leads to occurrence of their residues in milk.

2.2: Antibiotic residues in milk:

Antibiotics used as therapeutic agents or as feed supplement in milk animal lead to secretion of their residues into milk. These residues not only create problems in dairy industry but also have immense public health significance.

Hamann *et al*., (1979) reported that presence of antibiotic residues in milk is influenced by (a) concentration and type of antibiotic used, (b) carrier employed in the preparation, (c) amount of milk drawn from the gland, (d) time interval

between treatment and milking, (e) absorbance of udder tissue, (f) milk yield and (g) individual factors.

The antibiotic contamination of milk was reported to be due to intramammary infusions of drugs for mastitis treatment (92%), injections (6%), and other causes (2%) (Booth, 1982). In a study conducted by Brady and Katz, (1988) it was observed that 40% of milk samples were positive for tetracycline residues. Approximately 4.2 million milk samples were tested for antibiotics residues and 30.9 million kg of milk was dumped because of positive assay results (Gardner *et al*., 1996).

Lee *et al*. (1996) examined antibiotic residues in milk after intramuscular injection, intra-uterine injection and oral administration in dairy cows. Oxytetracycline residues were detected in milk after intramuscular injection. It was concluded that type of antibiotic, dosage and route of administration influenced the duration over which antibiotic residues could be detected in milk. It was further noted that milk yield on the sampling day and lactation stage had no influence on the duration over which antibiotic residues could be detected in milk.

Jarunee Loksuwan (2002) evaluated the effect of heating on the residues of oxytetracycline, tetracycline and chlortetracycline in milk. The residues were measured using high performance liquid chromatography with a UV detector. Milk spiked with OTC, TC and CTC at 200, 200 and 400 ppb respectively, were heated to 63^0C for 30 minutes. OTC residues were significantly ($p \leq 0.05$) reduced 79.36-86.77%. TC residues were significantly ($p \leq 0.05$) reduced 22.97-54.75%. No significant ($p > 0.5$) reduction of CTC was found. Results showed

that normal pasteurization procedure (63^0C for 30 min) causes a reduction in OTC, TC and CTC residues in milk, but it does not completely eliminate all the residues from milk.

Molina *et al.* (2003) analysed the main factors influencing antibiotic depletion time in lactating dairy sheep. A microbiological inhibition test (Brilliant Black Reduction, BRT) was used to screen all samples and antibiotic withholding times were established using a logistic regression model. The response to the BRT method in milk from individual ewes treated, showed that the effect of milking order was significant ($P < 0.001$) with the three antibiotics. However the only influence on milk yield was with the intramammary treatment ($P < 0.005$). The BRT method was found to be very sensitive, particularly to the two beta lactamic antibiotics.

2.3 Determination of tetracycline residues:

2.3.1 Microbial methods:

The earliest method used for detection of antibiotic residues in foods and foodstuffs was based on determining the inhibition of (sensitive) bacterial growth. These methods were based on inhibition of acid production or coagulation by starter cultures of bacteria.

Charm and Chi (1982) reported the introduction of charm test by penicillin assay incorporation, which was a product of modern biochemical technology. The principle of the test resembles Radio-Immuno Assay (RIA). The test uses two types of bacterial cells (*B. stearothermophilus* binding reagent) and a radiolabelled (^{14}C or ^{3}H) antibiotic (tracer reagent). The charm I and II tests are, in

fact, microbial receptor assays used for detection of antibiotic residues in milk and tissues. The technique was used for detection and identification of antimicrobial drug residues in milk. More than 70% of all U.S. raw milk samples were screened by Charm test.

Korsrud *et al*. (1995) applied charm Farm test for the detection of antibiotic residues in food and foodstuffs. The microbial growth inhibition method involved standard culture of *B. stearothermophilus*. The samples were incubated for a period of 3 hr. Negative samples turned green. The test was used to screen chlortetracycline, oxytetracycline, tetracycline, erythromycin, gentamicin, penicillin G, streptomycin and sulfamethazine antibiotics. The advantage of this method was that no expensive equipment is required and results could be obtained in a day.

Amosin *et al.,* (1996) screened nearly 1822 raw milk samples for detection of antibiotic residues by using *B. subtilis*. ATCC 6633, *B. stearothermophilus* var *caldolactis* C593 and Micrococcus luteus ATCC 9341 as standard test organisms. Of the total 51 (2.8%) samples were positive for antibiotic residues. Among the tested organisms, *B. stearothermophilus* var *calidolactis* was found to be more sensitive organism for detection of antibiotic residues.

Nouws *et al*. (1998) screened 973 samples of bulk milk with *B.calidolactic* tube diffusion test and receptor assay, which were Charm HVS and *B. cereus* ATCC 11778 mycoides test system. The *B. calidoactic* tube diffusion test detected oxytetracycline, tetracycline residues at concentration of 45 ng/ml. The *B. cereus* test plate detected oxytetracycline and tetracycline residues at

concentration of 30 ng/ml, while chlortetracycline and doxycycline limits were at 10ng/ml. Out of 973samples only one sample was positive by both the tests while 8 other samples were found positive by receptor assay alone. The *B. cereus* test was found more reliable and sensitive as compared to other receptor assay.

Althaus *et al*. (2002) reported the detection limit of 24 antimicrobial agents in ewe milk by Delvotest P. The test involved the use of standard culture of *B. stearothermophilus* var *calidolactis*. The method was found highly sensitive to penicillin, cephalosporins and sulphonamides but had low sensitivity for aminoglycosides, macrolides, tetracyclines, chloramphenicol and trimethoprim.

Althaus *et al.* (2009) reported about the application of a Microbiological Multi-Residue System in ewe milk, a method based on the use of six different plates, each seeded with one of the following bacteria: *Geobacillus stearothermophilus* var. *Calidolactis* (beta-lactams). *Bacillus subtilis* at pH 8.0 (aminoglycosides), *Kocuriarhizophia* (macrolides), *Escherichia coli* (quinolones), *B. cereus* (tetracyclines) and *B. subtilis* at pH 7.0 (sulphonamides), respectively. Twenty three antimicrobial substance were analysed and a logistic regression was established for each substance assayed to relate the antibiotic concentration and the zone of microbial growth inhibition. Great linearity was observed (regression coefficients of over 0.97).

Comunian *et al*. (2010) reported the evaluation of Delvotest Accelerator (DSM Food Specialties, Delft, the Netherlands), a new system for a fully automated microbial test to detect antibiotic residues in ewe and goat milk. 43 samples of raw, whole and refrigerated bulk tank milk samples (22 of ewe milk

and 21 of goat milk) were analysed during the whole lactation period. Four concentrations of 4 antibiotics were diluted in milk; penicillin G at 1, 2, 3, and 4 µg/L; sulfadiazine at 25, 50, 100, and 200 µg/L; tetracycline at 50, 100, 200 and 400 µg/L; and gentamicin at 25, 50, 100 and 200 µg/L. The detection limit of the Delvotest Accelerator was calculated at the range of antibiotic concentrations within which 95% of positive result lie. The range of detection limit of penicillin G and sulfadiazine was easily detected by Delvotest Accelerator at or below the European Union maximum residue limits, both for ewe and goat milk samples. Very low percentage of false positive outcomes were obtained.

Movassagh *et al*. (2011) collected 50 raw milk samples from Parsabad suburb milk collection centers from March 2009 to May 2009 by a systematic random sampling methods. Parsabad is located in Iran. All samples were examined Copan milk test (CHR Hansen, Denmark). Out of all samples 7(14%) were positive for antibiotic residues in cow raw milk in Parsabad region.

Syit (2011) conducted a cross sectional study to detect and determine oxytetracycline and penicillin G residue levels in bulk milk of cows in Debre Zelt dairy farms. A total of 400 milk samples were randomly collected and qualitatively screened for antibiotic residue by Delvotest SP assay. Concentration of the positive samples was determined by high performance liquid chromatography. Concentrations were established using linear calibration curves. Out of 400 samples analysed for antibiotic residue, 34 (8) milk samples were positive for antibiotic residues. The mean residue level of oxytetracycline was 142.0 µg/l and that of penicillin G was 4.77 µg/l.

Nagel *et al.* (2011) evaluated the Res Screen microbiological system for the identification of antibiotic residues, the BT (beta lactams and tetracyclines) and BS (beta lactams and sulphonamides) bioassays, containing spores of *G. stearothermophilus* subsp. Calidolactis, culture media and indicators (acid-base and redox). The detection limit of 25 antimicrobials agents were calculated using a logistic regression model. Both methods detected the residues of penicillin G, ampicillin, amoxicillin, cloxacillin, cephalexin, cefoperazone and ceftiofur at levels close to their Maximum Residue Limits (MRL). The BT bioassay also presented good sensitivity to tetracycline and oxytetracycline residues, whereas the as bioassay detected sulfadiazine, sulfamethoxazole and sulfathiazole residues in milk.

Nagel *et al*. (2012) reported a microbiological system for identification of antibiotic residues in milk. The "BT" bioassay contains spores of *Geobacillus stearothermophilus*, bromocresol purple and chloramphenicol in culture medium (incubation time: 2.45 h), while the "QS" bioassay uses spores of *Bacillus subtilis*, trifenyltetrazolium- toluidine blue and trimethoprim in a suitable culture medium (incubation time: 5.5 h). The detection capability (CC beta) of 27 antimicrobial agents in ovine milk were determined by logistic regression models. Thus, the "BT" bioassay detects amoxicillin, ampicillin, penicillin "G'', cloxacillin, oxacillin, cephalexin, cefoperazone, ceftiofur, chlortetracycline, oxytetracycline, tetracycline, neomycin, gentamycin and tylosin, while "QS" bioassay detects: ciprofloxacin, enrofloxacin, marbofloxacin, sulfadiazine, sulfadimethoxine, sulfamerazine, sulfamethoxazole, sulfathiazole, erythromycin, lincomycin and spiramycin at levels close to their respective Maximum Residue Limits.

Edima *et al*. (2012) assessed the contamination of raw milk by antibiotic residues, produced in Ngaoundere (Adamaoua region of Cameron). The results of survey revealed that the three antibiotics (oxytetracycline, penicillin and streptomycin) were routinely used. According to laboratory analysis, 27% of milk samples collected in farms was contaminated with antibiotic residues. Antibiotics of the beta lactams and tetracycline families (penicillin, oxytetracycline) was suspected to be the source of contamination of 53.85% of milk samples; whereas residues of macrolide and aminoglycoside antibiotics (streptomycin) were detected in 15.38% of the samples. A total of 30.77% of milk sample was found to be contaminated by residues o beta lactams and tetracycline and macrolide and aminoglycoside antibiotic families.

Salman *et al*. (2013) collected 734 stratified random raw milk samples from 47 dairy farms and sale points, to detect antibiotic residues and to compare between the Delvotest kit and Disc assay methods. The most frequent antibiotic used was Penicillin in 61.7% of the farms, while tetracycline was used in 27.7% of the farms. It was also found that antibiotic treated cows were milked together with the healthy ones. From the total number of samples tested using Delvotest Kit, 33.1% of samples were positive, out of which 42.4% were from the farms milk while 23.2% were from sale points. Milk samples were also tested using the two tests, 12.8% of the samples were positive in both tests , while 75.1% were negative in both tests.

Jabbar and Rehman, (2013) reported the use of microbiological tests to detect antibiotic residues in the meat, milk and eggs for better care of the quality and the health safety. In their study microbiological inhibition test i.e. Swab Test

on Animal Food (STAF) was developed indigenously for screening of animal foods for presence of antibiotic residues. In this test local isolated culture of *Bacillus subtilis* was used as a test microorganism due to its high sensitivity to detect a wide range of antibiotics commonly used in animal disorders. The concentration of spore suspension of *Bacillus subtilis* JS2004 used in the formation of STAF plate was optimized at 2×10^7 spores/ml. At this concentration, inhibition zone around Neomycin control disc was 10-16 mm. Nutrient agar was used as a medium in spore suspension and 0.4% dextrose was added as a constituent of medium. Zones of inhibition around swab samples and Neomycin control disc were observed and the diameter was measured. All swab samples showing a minimum of 2 mm wide inhibition zone around them were considered as positive for presence of antibiotic residues. The swab samples showing no zone of inhibition or a zone measuring less than 2 mm were considered as negative.

2.3.2 Chromatographic methods:

Chromatography is commonly used for separating components of a mixture. The identity and quantity of residues in a suspected sample cannot be determined with a screening test. Hence the decision about the compliance of a sample cannot be based upon a screening result. Therefore there is a need for specific chromatographic or other confirmatory methods.

At the beginning of the twentieth century, the Russian botanist Mikhail Tswett invented and named chromatography. He separated plant pigments by passing solution mixture through a glass column packed with fine particles of calcium carbonate. The separation of those pigments appeared as colored bands

on the column. Tswett named his separation method for the two Greek words "Chroma" and "graphein" which mean "color" and "to write," respectively (Skoog *et al*., 1998). In drug analysis chromatography was originally used to verify drug levels in formulations, serum and biological fluids for clinical applications. The 1952 Nobel Prize in chemistry was awarded to A. J. P Martin and R. L. M Synge for their contributions to chromatographic separations, which impacted chemistry related sciences. Between 1937 and 1972 a total of 12 Nobel Prizes were based on the work in which chromatography was key tool.

The chromatographic methods used in the residue analysis include gas chromatography (GC), thin layer chromatography (TLC), liquid chromatography (LC), high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC).

Bossuyt *et al*. (1976) devised a scheme that made it possible to separate and identify, by means of thin layer chromatography, 14 different antibiotic residues in milk which were, besides penicillin, the most widely used in mastitis control like, cloxacillin, dihydrostreptomycin, tetracycline, oxytetracycline, chlortetracycline, chloramphenicol, neomycin, novobiocin, bacitracin, erythromycin, oleandomycin, ampicillin, streptomycin and oxacillin. The limits of detectability of the antibiotics studied varied between 0.1 and 3 µg/ml, with the exception of neomycin, the minimum detectable concentration of which was 15 µg/ml.

Eksborg and Ekqvist (1981) studied reverse ion pair liquid chromatography of tetracyclines. They concluded that the retention of tetracycline was dependent on the acetonitrile, methanol and oxalic acid.

Oka *et al*. (1984a) reported that mixture of methanol: acetonitrile in the ratio 1: 1.5 yielded a good separation of oxytetracycline, chlortetracycline and tetracycline. A mobile phase of methanol: acetonitrile: 0.01 M aqueous oxalic acid solution was used in different proportions, and it was concluded that separation of tetracycline is dependent of pH of aqueous oxalic acid in mobile phase and optimum pH being 2.

Oka *et al*., (1984b) described semi-quantitative screening methods for tetracyclines using detection on silica gel high performance thin layer chromatography (HPTLC) and reversed phase (RP) TLC plates. Good results with respect to the background and detection limits were obtained using detection with 1% Fast Violet B Salt solution and pyridine without heating on the RP-TLC plate. The above detection method and UV densitometry using silica gel HPTLC plate were compared with respect to recovery from spiked samples after Sep-Pak C_{18}extraction. For the measurement of impurities in tetracycline drugs, both methods were compared using RP-TLC plate and similar results were obtained.

Tarbin *et al*. (1995) developed a liquid chromatographic (LC) method for the determination of benzyl penicillin (penicillin G) in milk. Milk was determined by centrifugation and deproteinised using sulphuric acid/sodium tungstate. Initial clean was achieved using C18 solid phase extraction (SPE). The SPE eluate was cleaned up by reversed-phase LC at acidic pH with post-column derivatisation

with imidazole/mercury (II) chloride reagent followed by UV adsorbance determination at 320 run. Average recoveries at 2 and 10 µg/kh were in the range of 70-73%. The lower limit of quantification was 2 µg/kg.

Valette *et al*. (2004) explored the potential of hydrophilic interaction chromatography (HILIC) is explored for the analysis of tetracycline antibiotics. It was shown that high efficiencies were reached only with a citrate buffer that impairs the interactions with the residual silanol groups whatever the mobile phase pH was. It was demonstrated that citrate buffer strongly interacts with the cationic moiety of the aminopropyl stationary phase and thus reduced the accessibility of silanols. The separation of oxyteracycline, tetracycline and chlortetracycline is achieved in a few minutes at pH 3.5 or 5, with no peak tailing as usually observed in reversed phase liquidchromatography with an opposite elution order when compared with reversed phase liquid chromatography.

Spisso *et al*. (2007) optimized and validated high-performance liquid chromatography-fluorescence detection method to determine tetracyclines residues in bovine milk. Post-column derivatization using metal complexation in non-aqueous reagent increased the fluorescence of chelates by a factor up to 2.54 compared to water (signal-to-noise ratio enhancement). Overall recoveries ranged from 61 to 115%, with RSD(r) from 5 to 15% (n=54). Detection limits ranged from 5 to 35 µg/kg. Limits of quantification were established at 50 µg kg. Decision limits (CCalpha) were 109, 108 and 124 µg/ kg and detection capabilities (CC beta) 119, 117 and 161 µg/ kg for oxytetracycline, tetracycline and chlortetracycline, respectively.

Fritz and Zuo (2007) reported about the reversed-phase high-performance liquid chromatography with photodiode-array detection (HPLC–PAD) for the simultaneous determination of tetracycline (TC), 4-epitetracycline (4-epiTC) and oxytetracycline (OTC) in milk. Milk samples were extracted and cleaned-up using solid-phase extraction Discovery SPE DSC-18 tubes. The separation were accomplished in less than 8 min in a Waters Symmetry C18 column at ambient temperature with a mobile phase consisted of 0.010 M aqueous oxalic acid: acetonitrile: methanol (150:20:20 by volume). Quantitation was carried out by the peak area method, with detection limits of 2.0 µg/l of each tetracycline. Average recoveries of TC, 4-epiTC and OTC from spiked samples at the four concentrations (0.25, 0.5, 1.0 and 1.5 µg/ml) were 91.5, 71.5 and 83.1, respectively, with their standard deviations less than 4% within a day and 7% between days. Oxytetracycline was found in all samples in a concentration range of 13–106 µg/l, 4-epiTC in most samples at 18–65 µg/l, TC in one sample at 44 µg/l.

Navratilova *et al*. (2009) detected the presence of tetracycline, chlortetracycline and oxytetracycline residues in raw cows milk. When analysing bulk milk (n = 57) and tanker trailer (n = 113) samples, two methods were used simultaneously: a specific rapid test Milk Tetra sensor Kit and high performance liquid chromatography (HPLC) with ultraviolet detection and isocratic elution. In all of the samples analyzed by means of HPLC, low concentrations of tetracycline antibiotics residues were detected. None of the samples displayed the presence of chlortetracycline. All of the analyzed samplesdisplayed residues of tetracycline. Oxytetracycline residues were detected only in 50.6% of analyzed samples.

Shahid *et al*. (2007) conducted study to monitor the status of oxytetracycline residue in poultry meat in Rawalpindi and Islamabad area of Pakistan. The preliminary screening of samples for the presence of antibiotic residue was performed by the microbiological assay using *Bacillus subtilis* a test organism. OTC in positive sample is detected and quantified using high performance liquid chromatography (HPLC). A linear calibration curve was obtained with correlation coefficient of 0.9981 while average recoveries was greater than 91% with RSD values between 1.64 to 2.07 while limit of detection was 0.01 µg/ml. Out of 29 meat samples that were analyzed for OTC residues 13(44.8%) had detectable residue level for OTC and 6(20.7%) had higher residue level than the recommended maximum residue level (0.2, 0.6 and 1.2 µg/gm) for muscles, liver and kidney, respectively.

Shariati *et al*. (2009) developed a simple and efficient pre-concentration method using carrier mediated three phase liquid phase micro extraction prior to HPLC-UV for simultaneous extraction and determination of trace amounts of highly hydrophilic tetracycline antibiotics including tetracycline, bovine milk, human plasma and water samples. Aqueous receiving phase (RP, 24 µL of 0.1 M $H_3PO_4$and 1.0 M NaCl with pH 1.6) was located inside the lumen of a hollow fiber and the fiber was transferred into the aqueous sample. Under the optimized conditions, the calibration curves were linear in the range of 0.5- 1000 µg/L for TC and OTC, and in the range of 5-1000 µg/L for DC with good linearity r^2>0.995).

Spisso *et al*. (2009) developed a liquid chromatography electro spray ionization tandem mass spectrometric (LC-ESI-MS/MS) method for the analysis

of several tetracycline residues in bovine milk. A central composite (response surface) design with desirability function was employed for the optimization of extraction and clean up steps. The optimization improved the extraction efficiency of the more polar analytes reaching 93.9% for 4-epioxytetracycline and 95.8% for oxytetracycline at 100 µg/L. The validation was performed following the criteria by Commission Decision 2002/657/EC.

Kishida *et al*. (2011) reported a simplified method for the simultaneous determination of oxytetracycline, tetracycline, chlortetracycline, and doxycycline in milk. Isolation of the target compounds was performed using an Ultrafree-MC/PL centrifugal ultrafiltration device without prior sample preparation. Analyses were carried out via high-performance liquid chromatography using a Discovery HS F5 column with a gradient mobile phase which consisted of 30 mM citric acid solution (pH 3, in water) and acetonitrile (60:40 to 40:60, v/v). Recovery of the target compounds from spiked samples at four levels (0.05, 0.1, 0.2, and 0.5 µg/mL) was higher than 87%, with relative standard deviations of less than 6%. Limits of quantification ranged from 0.01 to 0.04 µg/mL.

Abbasi *et al*. (2011) reported the presence of TCs residues in various bovine milk samples from local markets of Ardabil, Iran.Determination of TCs residues were performed by high performance liquid chromatography (HPLC) method using Fluorescence detector. Out of 114 samples, Twenty five point four percent of the all samples, and24.4%, 30% and 28.6% of thepasteurized, sterilized and raw milk samples, respectively had higher TCs residues than the recommended maximum levels (100ng/g).

Mishra *et al*. (2011) reported the effect of pasteurization on cloxacillin residue in milk. Milk samples were collected at day-0 (control), day-1, day-3 and day-5 after single IM administration of cloxacillin in 8 lactating cows. The milk samples were extracted with organic solvent and then separated by C18 RP-Column using an isocratic elution and detected with UV detector at 220nm. All 8 cows treated with cloxacillin showed significant level of residues concentration of cloxacillin on day-1. However, similar animals did not show residue concentration of cloxacillin in milk samples collected on day-3 and day-5. The effect of pasteurization (LTLT) at 65 0c for 30 min on cloxacillin residue in milk was also evaluated. No significant ($p>0.05$) reduction of cloxacillin residue in milk was found on pasteurization

Rao *et al*. (2012) reported a simple, sensitive and inexpensive method using matrix solid-phase dispersion (MSPD), together with high performance liquid chromatographic method for determination of antibiotic residues (lymecycline and tetracycline) in bovine milk. The best results were obtained using 2ml of milk sample, 1.0 g of C18 as sorbent and 20 ml of 20% trichloroacetic acid, 0.01 M citric acid, 0.01 M disodium hydrogen phosphate, 0.01 M EDTA and methanol (1: 2.2: 2.3, v/v). The method was validated using in milk samples spiked with antibiotic at different concentration levels (0.01 and 0.1 µg/ml). Average recoveries (using each concentration six replicates) ranged 88-96%, with relative standard deviations less than 2%, calibration solutions concentration in the range 0.01-2.0 µg/ml and limit of detection (LOD) and limit of quantification (LOQ) were 0.01 µg/ml and 0.03 µg/ml.

Kamberi *et al*. (2012) collected the oxytetracycline residues in cow's milk collected in farms of Tetovo in Macedonia. The cow's milk samples produced in this area are controlled applying qualitative analytical tests for oxytetracycline residues in 262 milk samples through specific ELISA test. After this control positive milk samples were kept in freezing conditions to be analyzed by high performance liquid chromatography (HPLC) method in order to perform qualitative evaluation of oxytetracycline. The quantitative control with ELISA confirmed that 5.3% (14/262) of milk samples were positive for oxytetracycline residues. From analytical check performed by HPLC, it was confirmed that 2.3% (6/262) of total milk samples had different values of oxytetracycline residues. Referring to MRL for oxytetracycline in milk only 1.6% (4/262) of analyzed samples has been confirmed with values higher than this limit. The quantity values of oxytetracycline calculated after analytical check with HPLC in 1 liter milk ranged 65 ug/l to 1300 ug/l (ppb).

Boultif *et al*. (2014) detected and quantified oxytetracycline residues in milk by high performance liquid chromatography. The results of the validation process led to the adaptation of following parameters: Sample concentration: 0, 4 mg/mL (diluted in methanol), stationary phase: column C18 reversed phase (Grafted silica gel), mobile phase: acetonitrile, injected volume: 11 µl, wavelength (UV detector): 325 nm and flow rate: 1, 5 mL/min. The drug were extracted with mixture acetonitrile/HPLC water and simply cleaned up on Whatman filter, then on a nylon membrane filter of 45 µm.

Tona and Olusola (2014) reported that out of 40 dairy product samples, all the analyzed samples contained residues of tetracycline antibiotics. This study

investigated the levels of the antibiotic tetracycline contained in cow milk, goat milk, butterfat, soft cheese and yoghurt samples in Oyo state Nigeria. High performance liquid chromatography (HPLC) standard methods were used for the detection and quantification of samples. The mean tetracycline residual levels were as follows: 0.0032 ± 0.0018 ppm (cow milk); 0.0040 ± 0.0011 ppm (goat milk); 0.0020 ± 0.0008ppm (butterfat); 0.0080 ± 0.0034 ppm (soft cheese) and 0.0019 ± 0.0008 ppm (yoghurt). The level of the tetracycline antibiotic in soft cheese was significantly ($P<0.05$) higher than those for cow milk, goat milk, butterfat and yoghurt, whose values were similar ($P>0.05$). The detection of tetracycline residues in all of the tested samples revealed that the processing of cow milk into milk products did not eliminate the tetracycline antibiotic.

Gupta *et al*. (2014) reported the validation of the method revealed that all obtained calibration curves showed good linearity ($r^2> 0.999$) over the range of 40-4500 ng. Sensitivity was found to be1.54 and 1.80 ng for oxytetracycline (OTC) and TC. Accuracy was in the range of 87.94-96.20% and 72.40-79.84% formeat. Precision was lower than 10% in all cases indicating that the method can be used as a validated method.Limit of detection was found to be 4.8 and 5.10 ng for OTC and TC, respectively. The corresponding values of limit of quantitation were 11 and 12 ng.

Kellnerová *et al.* (2014) reported the effect of high pasteurization of milk (85 °C/3 s) on the residues of tetracycline and oxytetracycline. The samples of raw cow's milk, purchased from a vending machine, were spiked with standard solutions of tetracycline and oxytetracycline. The content of the residues of tetracycline antibiotics was measured before and after heating. Pre cleaned

samples were extracted by a mixed-mode solid phase extraction technique and analyzed using high performance liquid chromatography/diode array detection. Whereas the residues of tetracycline decreased only by 5.74% and were not significantly different ($P > 0.05$), the residues of oxytetracycline decreased by 15.3% and this distinction was highly significant ($P \leq 0.01$).Based on the results of our study, the tetracycline antibiotics were proved to have differences in the thermo stability of particular substances at pasteurization temperatures.

Aalipour *et al*. (2015) reported the average concentration of total TETs in milk as 252.41 μg/kg, which is approximately 2.5 times greater than the maximum residue limit (MRL) set by codex. Of the four different tetracycline antibiotics analyzed, oxytetracycline had the highest share (86 %) of the determined contamination. To quantify the drug residues, HPLC analysis was performed under isocratic conditions using UV detection at355 nm.

Han *et al*. (2015) detected 38 veterinary antibiotic residues in raw milk by ultra-high performance liquid chromatography, the samples are extracted and purified using Oasis HLB cartridge. The results indicated recoveries of 68–118% for 14 b-lactams, 79–118% for eight quinolones, 71–106% for eight sulfonamides,76–116% for four tetracyclines, 78–106% for three macrolides, and 88–103% for one lincosamides.

2.4 Maximum residue limits:

A number of national and international organizations are involved in the legislation on residues of veterinary drugs in foods. Countries tend to follow their own guidelines. The Food and Agricultural organization (FAO) and World Health

Organization (WHO) have set up a joint FAO/WHO "Codex Alimentarius Commission" to coordinate food standards throughout the world. One of the main tasks of Codex Committee on residues of Veterinary drugs in food (CCRVDF) is to establish worldwide Maximum Residue Limits (MRLs). Other international groups active in this area include the European Agency for Evaluation of Medicinal Products (EAEMP), Office International des Epizootics (OIE) and Consultation Mondiale de I' Industrie de la Sante Animale (COMISA) (Mitchell *et al*., 1998). Several countries have the specialist groups, i.e., Food and Drug Administration (FDA), USA; Bureau of Veterinary Drugs, Canada and Veterinary Products Committee (Ministry of Agriculture, Fisheries and Foods), UK (Telling, 1990).

The limits of drug residues in foods have been established in the form of tolerances or maximum residue limits (MRLs). The term tolerance is used in United States while MRLs is used in Canada and European Union but these two terms are synonyms (Brynes and Yong, 1993). MRL is defined as maximum concentration of residues following administration of a veterinary medicine, which is legally permitted or acceptable in foods and foodstuffs. The MRL is based on the Acceptable Daily Intake (ADI) for that compound. The ADI is rough estimate of the amount of veterinary drug expressed on a body weight basis that can be ingested daily over a lifetime by a person without any appreciable toxicological risk (Brynes *et al*., 1996). The MRLs and ADI for tetracyclines (oxytetracycline, chlortetracycline and tetracycline) as recommended by Joint FAO/WHO Committee in Food Additives (2002) are 100 µg/litre of milk and 30 µg/kg body weight, respectively. MRL for tetracycline (oxytetracycline,

chlortetracycline and tetracycline) is 1200 µg/kg (kidney), 600 µg/kg (liver), 200 µg/kg (muscle) and 400 µg/kg (egg). The U.S Food and Drug Administration have set the tolerance of 0.3 mg/l for combined residues of TC, OTC and CTC.

MRLs have been determined by various committees and then included in legislation (Food and Drugs Act and Regulations in Canada, List of Codex MRLs for Veterinary Drugs, Official Journal of European Communities, Code of Federal Regulations in the United States) for animal products such as meat, eggs and milk (Code of Federal Regulations, 1994, Codex Alimentarius Commission 1993)

Withdrawal period is the time between the last recommended treatment and time of slaughter (meat) or collection for use as foods (milk and egg). This time allows the veterinary drug and its residues to decrease to levels below the established MRL. Until the withdrawal period has elapsed the animal or its products are not fit for human consumption. It varies with each drug preparation and target animals. Depending upon the drug, products, dosage, and route of administration, it varies from a day to several days or weeks (Lee *et al*., 2001). The involvement of many organization legislation of veterinary drugs has made it very difficult to standardize control practices and harmonize tolerance levels internationally in an uniform manner. The differences in tolerance levels are mainly due to differences in the use of compounds, food habits, choice of safety factors and food consumption values (Brynes and Yong, 1993). Therefore, it has been proposed that the ADI is better choice for determination of food safety rather than MRL (Brynes *et al*., 1996).

2.5 Public health significance of antibiotic residues in milk

As per IDF (1995) drug or antibiotic residues are remnants of antibiotic drugs or their active metabolites that are present within tissues or products e.g. meat, milk and eggs from treated animals. The presence of antibiotic residues and its associated harmful health effects on consumers makes the control of antibiotic residues an important measure in ensuring consumer protection. Concern over antibiotic residues in foods of animal origin occurs in two times; one which produces potential threat to direct toxicity in human, second is whether the low levels of antibiotic exposure would result in alteration of microflora, cause disease and the possible development of resistant strains which cause failure of antibiotic therapy in clinical situations (Butaye *et al*., 2001). In addition consumption of trace levels of antibiotics residues in food may have consequences in the indigenous intestinal micro flora which constitutes an integral component of human physiology. This flora act as barrier against colonization of gastrointestinal tract by pathogenic bacteria (Vollard and Clasener, 1994) and has an important role for food digestion. So, the ingestion of trace level of antimicrobials must take into account potentially harmful effects on human gut flora. The withdrawal time is the time required for the residue of toxicological concern to reach safe concentration as defined by tolerance. The presence of residues may result from failure to observe the mandatory withdrawal periods, illegal or extra-label use of drugs and incorrect dosage.

Symptoms of chronic exposure of oxytetracycline includes blood changes such as leucocytosis, atypical lymphocytes, lung congestion, toxic granulation of granulocytes and thrombocytopenia purpura. Liver injury and delayed blood

coagulation can also occur. It can damage calcium rich organs such as teeth and bones and sometimes causes nasal cavities to erode. Children under 7 years of age may develop to discolouration of the teeth. Infants of mother treated with oxytetracycline during pregnancy may develop discoloration of the teeth. Some other chronic effects of oxytetracycline include increased sensitivity to the sun, wheezing, asthmatic attack and allergic anaphylactic reactions. Toxicological studies indicate that this drug is not mutagenic, carcinogenic and teratogenic. Sixty percent of ingested dose absorbed from gastro-intestinal tract and widely distributed in the body, particularly to liver, kidney, bones and teeth.

Antibiotic usage in veterinary practice may impact human health because animals, can serve as mediators, reservoirs and disseminators of resistant strains/or AMR genes. Consequently, imprudent use of antibiotics antimicrobials in animals may unnecessarily result in increased human morbidity, increased human mortality, reduced efficacy of related antibiotics used for human medicine, increase health care cost, increase potential for carriage and dissemination of pathogens within human populations and facilitated emergence of resistance human pathogens.

Barton (2000) suggested that resistance in some enteric pathogens has arisen because of transfer of resistant genes from animals through food chain. The resistant strains of staphylococci, coliforms, bacilli, pneumococci, hemolytic streptococci, and *Clostridium welchii* have been encountered. Aarestrup (1995) reported isolation of vancomycin resistant enterococci (VRE) from pigs and chickens fed avoparcin.

Moats (1988) emphasized on the safety of human food, which is threatened by various agents including pathogenic microorganisms, aflatoxins, pesticides and antimicrobial agents. Pathogenic microorganisms constitute the most important food related threat to public health. Relatively, little is known about food safety in relation to microbial agents. It was further emphasized that while pasteurization and other forms of heat treatments eliminate pathogenic microorganisms from animal source food. These procedures have limited or variable effects on drug residues in animal originated food.

Myllyniemi *et al*. (2000) reported about the disruptive potential of drug residues in human food on the normal human flora in the intestine. The bacteria that usually live in the intestine act as barrier to prevent incoming pathogenic bacteria from becoming established and causing disease. It was emphasized that antibiotics might reduce total no of the benign bacteria of selectively kill some important species.

Donoghue (2003) reported that both the FDA and USDA provide extensive regulatory oversight to ensure the safety of food supply. This included mandatory and safety (toxicology and pharmacokinetic) studies prior to the approval of an antibiotic for use and monitoring of the food supply to ensure the antibiotic is being used correctly. Furthermore, the federal government, universities and private research facilities conducted that allows for monitoring antibiotics that may be used illegally.

WHO/FAO/OIE (2003) concluded in an expert workshop co-sponsored by the World Health Organization, Food and Agricultural organization (FDA) and

World Animal Health Organization (OIE), 'that there is clear evidence of adverse human health consequences due to resistant organism resulting from non-human uses of antimicrobials. These consequences included infections that would not have otherwise occurred increased frequency of treatment failures (In some cases death) and increases severity of infections."

Nisha (2008) reported the fact that antibiotic residues in milk that was used to produce fermented products can interfere with the fermentation process by effecting desired lactic acid bacteria. Normally, this is just a technical problem resulting financial loss, but, when it occurs pathogens present in the milk may grow and pose a health hazard later. For these reason many countries have regulations prohibiting the sale of milk from cows being treated for mastitis and milk is routinely tested for the presence of antibiotic residues.

Jeong *et al*. (2010) reported that small amounts of antibiotics added to feedstuff present growth promotion effects via the prevention of infectious diseases at doses lower than therapeutic dose. The disruption of normal human intestinal flora is major concerns in terms of human health impact. Regulatory guidance such as ADIs and MRLs fully reflect the impact on human gastrointestinal microflora. However, before deciding on any risk management options, risk assessments of antimicrobial resistance require large-scale evidence regarding the relationship between antimicrobial use in food-producing animals and the occurrence of antimicrobial resistance in human pathogens.

Anthony *et al*. (2010) reported that many scientists, activists, regulators, and politicians have expressed urgent concern that using antibiotics in food

animals selects for resistant strains of bacteria that harm human health and bring nearer “post antibiotic era” of multidrug resistant “super-bugs.” Proposed political solutions, such as the Preservation of Antibiotics for Medical Treatment Act (PAMTA), would ban entire classes of subtherapeutic antibiotics (STAs) now used for disease prevention and growth promotion in food animals. The proposed bans are not driven by formal quantitative risk assessment (QRA), but by a perceived need for immediate action to prevent potential catastrophe. As a case study, examining specific tetracycline uses and resistance patterns suggests that there is no significant human health hazard from continued use of tetracycline in food animals. Simple hypothetical calculations suggest an unobservable small risk (between 0 and 1.75E-11 excess lifetime risk of a tetracycline-resistant infection), based on the long history of tetracycline use in the United States without resistance-related treatment failures.

Vragovic *et al*. (2011) assessed the quantitative risk of streptomycin and tetracycline. The median values for streptomycin In milk and meat was 11.50 and 38.00 µg/kg, respectively (milk; average: 15.57 µg/kg : range from 0 to 73.82 µg/kg, meat; average: 44.14 µg/kg: range from 0 to 278.35 µg/kg). Based on the median values it was concluded that the estimated daily intake of streptomycin and tetracycline through milk and meat in Croatia was low (streptomycin: 7.33 µg/person/day; tetracycline: 0.52 µg/person/day), and the risk was assessed as negligible.

Hsieh *et al.* (2011) reported about the heat stability of 14 veterinary antibiotics under a short-term heating scenario by characterization of their structural degradation and their relationship to resultant changes in antimicrobial

activity. Mutagenicity was also examined in four representative antibiotics after 15-min-heat treatments at two temperatures (100 °C and 121 °C). Heat treatment resulted in the reduction of the main peak and the production of new peaks in certain antibiotics. Ranking of heat stability by antibiotic classes at 121 °C was highest for sulfonamides, followed by lincomycin, colistin, tetracyclines and β-lactams while at 100 °C sulfonamides equaled lincomycin and was greater than colistin but variability was observed within different tetracyclines and β-lactams. The markedly variable heat stabilities within the classes of tetracyclines and β-lactam antibiotics highlighted the fact that heat stability within these two classes can be very different despite their structural similarity. Mutagenicity (Ames) tests on heated chlortetracycline (CTC) resulted in 2- to 6-fold revertant changes in Salmonella typhimurium TA98 and TA100. The combined results suggest that correlation analysis of structural degradation and antimicrobial activity offers dual evaluation of a drug's heat stability but gives little advantage over assessment of the resultant toxicity.

Boonsaner and Hawker (2013) reported the results of the bioconcentration of the antibiotic oxytetracycline by the Asian watermeal plant (*Wolffiaglobosa* Hartog & Plas) and bioaccumulation of OTC in watermeal and water by the seven-striped carp (*Probarbus jullieni*). They show, for the first time, the extent to which OTC is able to transfer from water to plant to fish and enter the food chain. The bioconcentration and biomagnification factors for these processes were 1.75 L/kg and 2×10^{-4} kg/g respectively. Using an aqueous concentration range of 0.34-3.0 μg/L, hazard quotients for human consumption of contaminated fish of 1.3×10^{-2} to 1.15×10^{-1} were derived.

Interagency Task Force on Antimicrobial resistance (2012) stated that antibiotics have been used since the 1940s and have led to dramatic reduction in illness and death from infectious diseases. According to them, "The extensive use of antimicrobial drugs has resulted in drug resistance that threatens to reverse the medical advances of the last 70 years."

Zaitseva *et al.* (2014) reported health risk from tetracycline residues in food, as one of the most widespread veterinary antibiotic. Health risk assessment of veterinary drugs residues in food in particular within the World Trade Organization, the Eurasian Economic Community and the Eurasian Economic Community customs union is one of the priority areas in the field of consumer health safety. According to results of gut flora alterations modeling for children it was founded that tetracycline residues concentration in food more than 10 μg/kg increases risk of digestive system diseases to 0.000461 (up to 4% of cases), risk of dermatitis to 0.000725 (up to 0.9% of cases), risk of alimentary allergy to 0.000149 (up to 0.1% of cases), risk of diseases of the blood to 0.001372 (up to 8% of cases). Health risk assessment on tetracycline in food showed that tetracycline residues at 10 μg/kg (allowable residue level for Customs Union members) led to no health risk increase including most sensitive population.

Padol *et al.* (2015) stated that antibiotic usage in livestock production as therapeutics, prophylactics and as growth promoter has become vital to the growing dairy industry, since the prolonged or inappropriate usage of such antibiotics may lead to residues appearing in milk, which pose the risk of human health hazards and also interfere with the processing of the milk and milk products. The administration of antibiotics against bacterial infection is a significant driving

force for selection of resistant strains of bacteria, which can spread from animal to human population and complicate the therapeutic management of such infections. Despite the numerous investigations performed, there is still a lack of understanding and knowledge about antibiotic residues in the milk and milk products. In this review authors addressed the present state of knowledge concerning the occurrence, fate, public health hazards and the methods used to detect of antibiotic residues in milk.

Peiyuan *et al.* (2015) investigated the multiple scattering correction method for pretreatment, chicken spectral information was collected by near infrared spectrometer. The forecast model was built by interval partial least squares regression method to analyze the tetracycline quantitatively. Then, according to the data of intake by human body dietary with tetracycline residues, the dietary exposure was calculated so as to introduce food safety index indicators to assess the health risk from the chicken. Based on risk assessment model combined with the national standard, the chicken risks divided into high, medium and low levels, the accuracy rate of hierarchy could reach to 95.5%.

Aalipour *et al.* (2015) investigated the average concentration of total TETs in milk was determined to be 252.41 μg/kg, which is approximately 2.5 times greater than the maximum residue limit (MRL) set by codex. Of the four different tetracycline antibiotics analyzed, oxytetracycline had the highest share (86 %) of the determined contamination. Daily exposure to TETs through milk using an average data on milk consumption was estimated to range from 58–62 μg, but, distribution based exposure to TETs in milk appeared as 0–99.3 μg/day. Risk characterization of dietary exposure to TETs residue via milk intake in different

age groups showed that considering the standard dietary recommendation that advices on two servings of milk per day (480 ml), consumers may receive 7–30 % of the determined ADI via bovine milk consumption.

2.6 Antibiotic residues and emergence of antibiotic resistance

Alali *et al*. (2009) reported a longitudinal ecological study to examine the e the relationship between the prevalence of antibiotic-resistant (AR) commensal Escherichia coli isolates from both monthly human wastewater and composite swine fecal samples. Human and swine *E. coli* isolates (n = 2469 human and 2310 swine, respectively) were tested for antimicrobial susceptibility using a commercial broth microdilution system. The relative odds of ciprofloxacin resistance were significantly increased for ciprofloxacin use in non-swine workers (OR = 5.5) as compared to the referent (non-use). The relative odds of tetracycline resistance were increased significantly for chlortetracycline use in medicated feed for the upper tertile of MMD category (OR = 2.9) as compared to the referent category (no use) across all swine production groups

Cho *et al*. (2012) compared the antibiotic resistance of *Escherichia coli* isolates from faecal samples of workers who often used antibiotics. A total of 163*E coli* strains were analyzed by agar disc diffusion to determine their susceptibility patterns to 16 antimicrobial agents. Most of the tested isolates showed high antimicrobial resistance to ampicillin and tetracycline. The isolates showed higher resistance to cephalothin than other antibiotics among the cephems. Among the aminoglycosides, the resistance to gentamicin and tobramycin occurred at higher frequencies compared with resistance to amikacin and netilmicin. Data indicated

that faecal *E coli* isolates of livestock workers showed higher antibiotic resistances than non-livestock workers (restaurant workers), especially cephalothin, gentamicin, and tobramycin ($p < 0.05$).

Ji *et al*. (2012) quantified Eight antibiotic resistance genes (ARGs), 7 heavy metals, and 6 antibiotics were quantified in manures and soils collected from multiple feedlots in Shanghai. The results revealed the presence of chloramphenicol, sulfonamides and tetracyclines at concentration ranges of 3.27-17.85, 5.85-33.37 and 4.54-24.66 mg kg(-1), respectively. Overall, sulfonamide ARGs were more abundant than tetracycline ARGs. Except for *sul*II, only a weak positive correlation was found between ARGs and their corresponding antibiotics. On the contrary, significant positive correlations ($p<0.05$) were found between some ARGs and typical heavy metals.

Gao *et al*. (2012) conducted a study in which total concentration of tetracycline and sulfonamide antibiotics in final effluent were detected at 652.6 and 261.1ng/L, respectively, and in treated sludge, concentrations were at 1150.0 and 76.0μg/kg dry weight (dw), respectively. The gene abundances of tetO and tetW normalized to that of 16S rRNA genes indicated an apparent decrease as compared to sulI genes, which remained stable along each treatment stage. Significant correlations ($R(2)=0.75-0.83$, $p<0.05$) between numbers of resistant bacteria and antibiotic concentrations were observed in raw influent and final effluent. No significance ($R(2)=0.15$, $p>0.05$) was found between tet genes (tetO and tetW) with concentration of tetracyclines identified in wastewater, while a significant correlation ($R(2)=0.97$, $p<0.05$) was observed for sulI gene and total concentration of sulfonamides.

Novo *et al*. (2013) reported about a study in which raw and treated wastewater composite samples were collected from an urban treatment plant over 14 sampling dates. Samples were characterized for the i) occurrence of tetracyclines, penicillins, sulfonamides, quinolones, triclosan, arsenic, cadmium, lead, chromium and mercury; ii) antibiotic resistance percentages for tetracycline, sulfamethoxazole, ciprofloxacin and amoxicillin and iii) 16S rRNA gene-DGGE patterns. Antibiotic resistance percentages presented different trends of variation in heterotrophs/enterobacteria and in enterococci, varied over time and after wastewater treatment. Antibiotic resistance was positively correlated with the occurrence of tetracyclines residues and high temperature.

CHAPTER- III

MATERIALS AND METHODS

Tetracyclines produced by *Streptomyces spp*. are broad spectrum antibiotics active against most Gram positive and Gram negative bacteria. In bovine, oxytetracycline, tetracycline and chlortetracycline are the major drugs of choice for prevention and treatment of bacterial infection. Because of their wide application, there is a concern about the presence of residues in milk. The detection of TCs residue is generally done by HPLC coupled with a suitable detector such as UV and florescence detectors. In present study, reversed-phase-high-performance-liquid-chromatography method coupled with photodiode-array-detector (PDA) was used for separation and quantification of tetracycline residues.

The study was undertaken to assess the status of Tetracyclines (Oxytetracycline, Tetracycline, Chlortetracycline) residues in milk in four district of Chhattisgarh.

The work was undertaken at the Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences and Animal Husbandry, Anjora, Durg, Chhattisgarh.

3.1 Chemicals

Methanol, acetonitrile, citric acid monohydrate and sodium chloride were of HPLC grade, were procured from Fisher Chemical and acetonitrile were procured by Himedia (Mumbai). EDTA disodium salt dihydrate and Oxalic acid

dihydrate were of analytical grade and obtained from Himedia (Mumbai) and Merck, respectively. GR grade Sodium phosphate Dibasic Dihydrate (Na_2HPO_4) was supplied by Loba chemie. Pure standard of oxytetracycline (assay 94.9% in HPLC), tetracycline (assay 98% in HPLC) and chlortetracycline (assay 93.3% in HPLC), as their hydrochloride, were obtained from Sigma-Aldrich Pvt. Ltd., (USA). Water for HPLC was obtained from Millipore water purification system. Mcllvaine-EDTA-NaCl buffer prepared according to the method of AOAC and filtered through 0.45 µm filter paper and stored at 4°c.

3.3 Apparatus

Vortex Shaker(Tarsons), Refrigerated Centrifuge (ThermoFisher), Electronic balance (Sartorius), Deep freezer (Remi), Hot air oven (Unitech), Refrigerator (LG), Ultrasonic bath (PCi Analytics) and filtration chamber with pump for vacuum creation were used during the course of present study.

HPLC system : Waters Alliance ® HPLC – e2695 Separation Module, Auto sampler with injector having a 100 µl loop, Waters ® 2998 Photodiode Array (PDA) Detector, Waters Alliance Series Column Heater/Cooler, Sample heater/cooler , Sample Syringe. HPLC Column: Waters C18 Sunfire column with the particle size of 5µm, which have 4.6 mm internal diameter and 250 mm column length.

3.4 Glass wares and plastic wares

The glass wares used in this study were procured from Borosil Glass wares Ltd. India, whereas, plastic wares and other disposables were procured from Tarsons Product Pvt. Ltd. India

3.4.1 Preparation of glasswares and plastic wares

All the glass wares and plastic wares were properly washed with detergent solution and kept overnight in distilled water and lastly safely dried in hot air oven prior to use.

3.5 Sampling

3.5.1 Study Area

Chhattisgarh is a state in central India. It is the 10th largest state, with an area of 135,194 km^2. About 80% of the population of the state is rural and the main livelihood of the villagers is widely based on animal husbandry practices to meet their demand. Raipur, Durg, Rajnandgoan and Balod are the areas where large numbers of dairies are situated practicing animal husbandry, thus milk samples were collected from these districts.

3.5.2 Collection and transport of milk samples

Milk samples from livestock owners and dairy units were collected from Durg, Rajnandgaon, Raipur and Balod districts of Chhattisgarh. Milk samples were collected in a clean sample collection container. Name of the owner and if any treatment given to the animal were properly noted. After notation of sample characteristics they were transported to laboratory in thermo-cooled container jackets with ice as soon as possible and were stored in -20ºc until analysed. The details of animal and their owner were recorded.

Table: 1 Samples collected from various districts of Chhattisgarh state

S.N	District	Cow milk Sample	Buffalo Milk sample	Total
1.	Durg	25	25	50
2.	Balod	25	25	50
3.	Rajnandgaon	25	25	50
4.	Raipur	25	25	50
	Grand total	100	100	200

3.6 Sample preparation

All milk samples were brought to the room temperature and were mixed thoroughly before tetracycline extraction and from that a representative sample was taken for extraction of tetracyclines.

3.6.1 Sample Extraction

Tetracyclines residues (OTC, TC and CTC) from milk samples were extracted as per the method described by Thomas (1989) with some modifications.

3.7 Preparation of standard solutions

(a) Stock standard solutions were prepared separately by dissolving 25 mg of each standard in 25 mL methanol and mixed properly. These stock solutions were stored in amber coloured bottles at -20ºc and used for a duration of 2 months.

(b) Second stock solutions of OTC, TC & CTC were prepared by dissolving 10 µl of stock standard solution of OTC and TC in 4990 µl millipore water and 150 µl stock standard solution of CTC in 4850 µl millipore water to make final volume to 5ml each and stored in separate amber coloured bottles in refrigerator for future use.

(c) Working solutions of standards were prepared on the day of its analysis by proper dilution of second stock solution with Mcllvaine-EDTA-NaCl buffer which was prepared by dissolving 12.9 g Citric acid monohydrate and 10.9 g anhydrous Sodium phosphate dibasic dihydrate in distilled water. The volume was made to one litre with water. Mcllvaine buffer was stored in refrigerator. Mcllvaine-EDTA-NaCl buffer was prepared by adding 37.2 g EDTA disodium salt dihydrate and 29.2 g Sodium chloride to 1litre volumetric flask and diluted with Mcllvaine buffer to the final volume of 1000 ml. The solutions was filtered with 0.45 µm PVDF membrane and stored in refrigerator. Second stock solution

of standards were properly diluted with buffer to made a concentration of 20-335 ppb for OTC and TC and 300-5070 ppb for CTC. From these solutions 100µl aliquots were used for the construction of calibration curves.

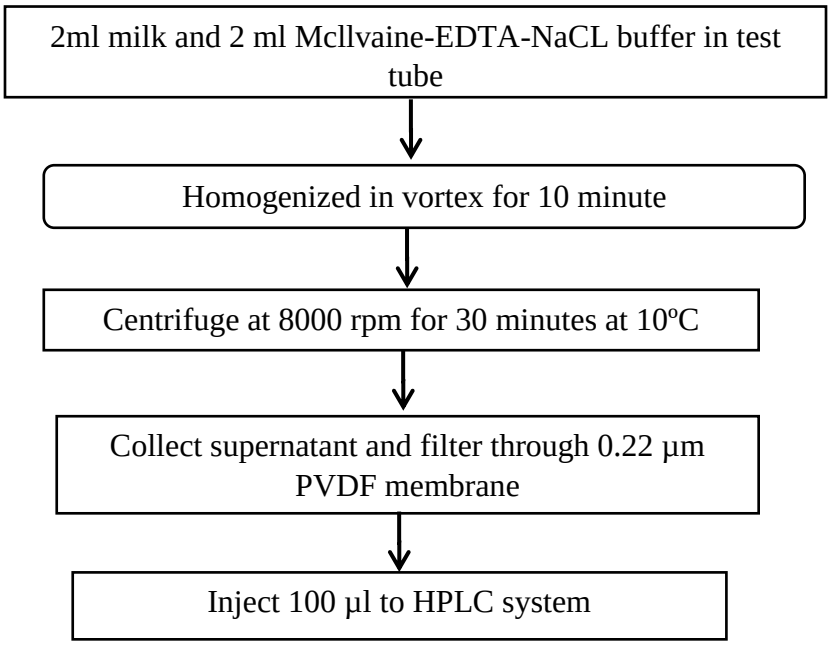
3.6.1.3 Blank Milk Samples

Amul milk sample after proper analysing for absence of tetracyclines in HPLC system was taken as blank milk sample.

3.6.1.4 Extraction procedure

Two ml of test milk sample was added with 2 ml of Mcllvaine-EDTA-NACL buffer in 50 ml centrifuge tubes. The mixture was mixed on a vortex shaker for 10 minutes for properly mixing of buffer and test milk sample and thereafter centrifuged at 8000 rpm for 30 minutes at 10°C in refrigerated centrifuge machine. The supernatant was collected and filtered through a 0.22 µm PVDF membrane filter into vials for HPLC analysis.

Flow chart: 3.1 Extraction of tetracycline residues (OTC, TC, CTC) from milk



3.7 Detection and estimation of Tetracyclines (OTC, TC, CTC) from milk

High performance liquid chromatography methods are becoming very popular for determination of wide variety of antibiotics drug residue from milk.

During the present study the HPLC system (Waters Alliance ® HPLC – e2695 Separation Module) with auto sampler, Quaternary LC Pump, Photo Diode Array Detector (PDA) and Column Oven with reverse phase column (Waters SunFire C18 with the particle size of 5µm, which have 4.6 mm internal diameter and 250 mm column length) was used for the analysis of antibiotic residues.

3.7.1 HPLC conditions:

Separations was achieved by gradient elution using mobile phase:

(A) Acetonitrile

(B) Methanol: Acetonitrile: 0.01 M oxalic acid (1:1.5:4.5), and a flow rate of 1mL/min. Further a gradient program 0A-100B to 35A-65B in 11 minutes and this composition is maintained for 2 minutes followed by return to initial condition in 2 minutes and maintained at initial condition for 5 minutes. So a total of 20 minute gradient run program were followed. All separations were done at 25ºC using Photo Diode Array Detector (PDA) set at 350 and 375 nm and the range of PDA wavelength was kept at 210-400 nm. The chromatograms were analyzed using 'Empower3' software

. 3.7.2 Standard calibration curves for the detection of OTC AND TC

Standard calibration curves of OTC and TC were prepared by mixing second stock solution in the range 20, 65, 110, 155, 200, 245, 290 and 335µl and Mcllvaine-EDTA-NaCl buffer in the range 1980, 1936, 1890, 1846, 1800, 1755, 1710 and 1665 µl respectively to obtain a final concentration of 20-335 ppb. An

aliquot of 100µl of these concentration were injected into the HPLC system and a standard curve was obtained by plotting concentrations versus the peak areas.

3.7.3 Standard calibration curves for the detection of CTC

Standard calibration curves of Chlortetracycline were obtained by mixing second stock solutions in the range 20, 64, 110, 154, 200, 246, 292, 338 µL and Mcllvaine-EDTA-NaCl buffer in the range 1980, 1936, 1890, 1846, 1800, 1754µl, 1708, 1762 µL to obtain a final concentration of 300-5070 ppb. An aliquot of 100 µl of these concentration were injected into the HPLC system and a standard curve was obtained by plotting concentrations versus the peak areas.

3.7.4 Method validation

To validate the multiple residue analysis method for OTC, TC and CTC in present study, the recovery of antibiotic residues was calculated by employing following formula :

$$\text{Recovery \%} = \frac{(xy)- (x)\,(y)\ \times 100}{N\,(x^2) - (x)^2}$$

Where,

x = Amount of standard antibiotic

y = Amount of antibiotic detected in present study

N = Total Number of Observations.

3.7.5 Correction Factor

Correction factor (*C.f.*) for a particular residue was calculated by the following formula for each of the residual concentration (Leoni *et al*., 1992)

$$C.F = \frac{100}{\text{Percent recovery}}$$

3.7 Statistical analysis

In the present study, the statistical analysis of data was done by estimating mean as a measure of central tendency and range as a measure of dispersion. Values of ranges and means of antibiotics in test samples were calculated.

CHAPTER-IV

RESULTS AND DISCUSSION

In the modern animal husbandry practice, antibiotics are being used in a large scale for different reasons. Tetracyclines are the broad spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria. These are used routinely in veterinary medicine for treatment and control of diseases. Tetracyclines is on the World Health Organization's List of Essential Medicines, a list of most important medications needed in a basic health system. However, misuse of these molecules results into the presence of their residues in foods of animal origin. Nowadays, in addition to the adverse effects that can occur as a result of the use of veterinary drugs, antibiotic resistance is considered to be major threat to human health.

In the present study, attempts were made to develop simple, sensitive, reproducible method of extraction, detection and quantification of tetracyclines viz. oxytetracycline, tetracycline and chlortetracycline residues in cow and buffalo milk. These methods were standardized for screening of 200 samples of milk collected from the four districts of Chhattisgarh.

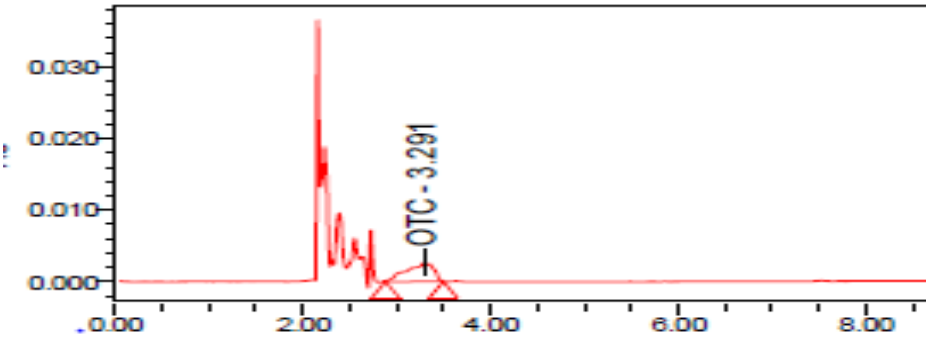
4.1 Optimization of HPLC conditions for the detection of tetracycline residues in milk

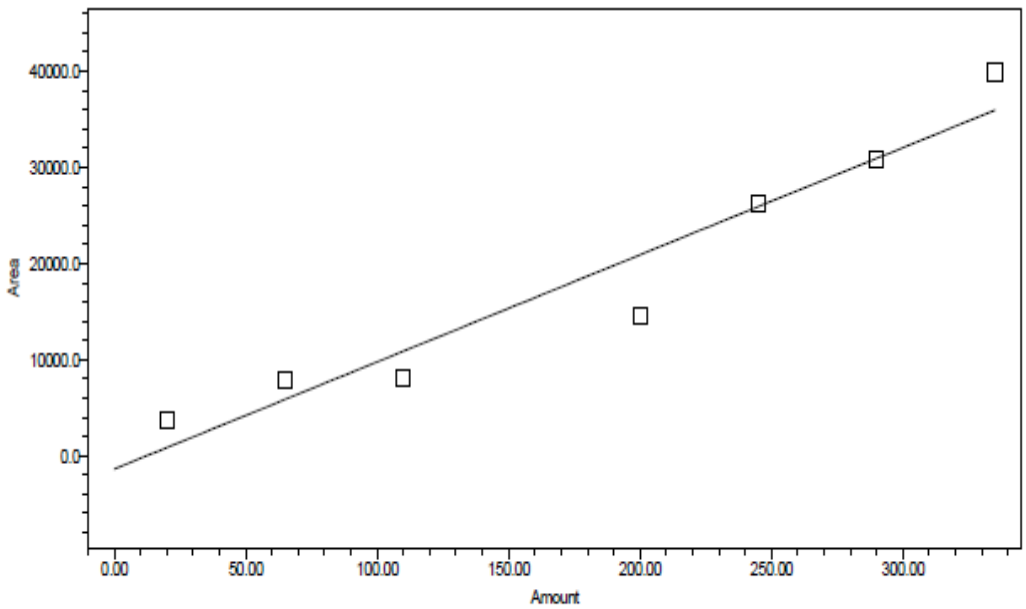
With an objective to develop simple, reproducible and cost–effective method for the sample extraction, the procedure described by Thomas (1989) were used with some modifications. The milk samples were screened for the presence or absence of residues of oxytetracycline, tetracycline and

chlortetracycline. Two ml of Mcllvaine-EDTA-NaCL buffer was mixed with 2 ml of test milk sample and subjected to homogenization for 10 min in vortex shaker, after proper mixing centrifugation was done for 30 min at 10^0C and after that supernatant was collected and filtrated using syringe filter of 0.22 µm PVDF membrane and lastly it was taken in vials for HPLC analysis. A gradient mobile phase of (a) acetonitrile and (b) methanol: acetonitrile: 0.01 M oxalic acid (1:1.5:4.5) composition was used. A gradient program 0A-100B to 35A-65B in 11 minutes was set and this composition was maintained for 2 minutes followed by return to initial condition in 2 minutes and maintained at initial condition for 5 minutes was used to elute the tetracycline residues. The flow rate was kept at 1 ml/min. Chromatography was performed at column temperature 25^0 C using Photo Diode Array Detector (PDA) for detection and estimation of tetracycline residues at 375 nm and the range of PDA wavelength was kept at 210-400 nm.214 nm. A 100 µl of injection volume was used for tetracycline analysis in the present study. Data processing was performed by Empower3 software. The standard calibration curve, obtained by plotting concentration against the peak area showed good correlation coefficient (R^2) of 0.9329 for OTC and TC and 0.998 for CTC respectively. (Fig. 1-3)

The findings of the present study are in accordance with Loksuwan *et al*., (2002). The mixtures of methanol: acetonitrile: oxalic acid in different proportions for the elution of tetracycline residues in milk samples with C18 column in ambient or higher temperature with a flow rate of 1ml/min were used by several researchers (Abbasi *et al*. 2011, Wen *et al*. 2006).

	Name	RT	Area	Height	Amount	Units
1	otc	3.293	39928	2924	335.000	=ppb
2	otc	3.361	30906	2408	290.000	=ppb
3	otc	3.347	26162	2012	245.000	=ppb
4		3.235	4394	895		
5	otc	3.164	3655	351	20.000	=ppb
6	otc	3.293				
7		3.235	3152	580		
8	otc	3.339	8146	903	110.000	=ppb
9	otc	3.277	7959	558	65.000	=ppb
10	otc	3.347	14594	1573	200.000	=ppb

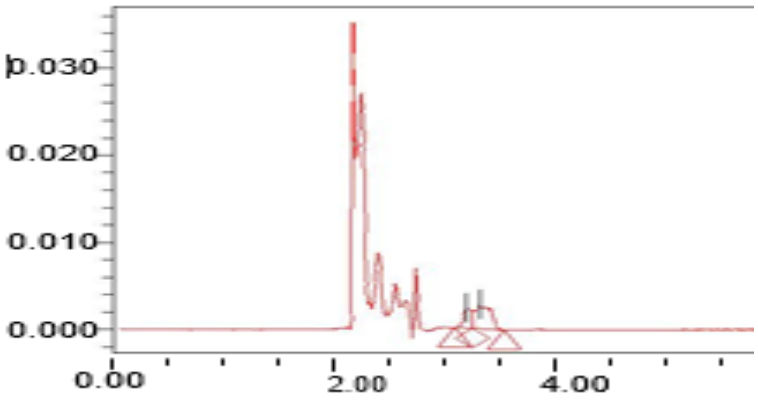


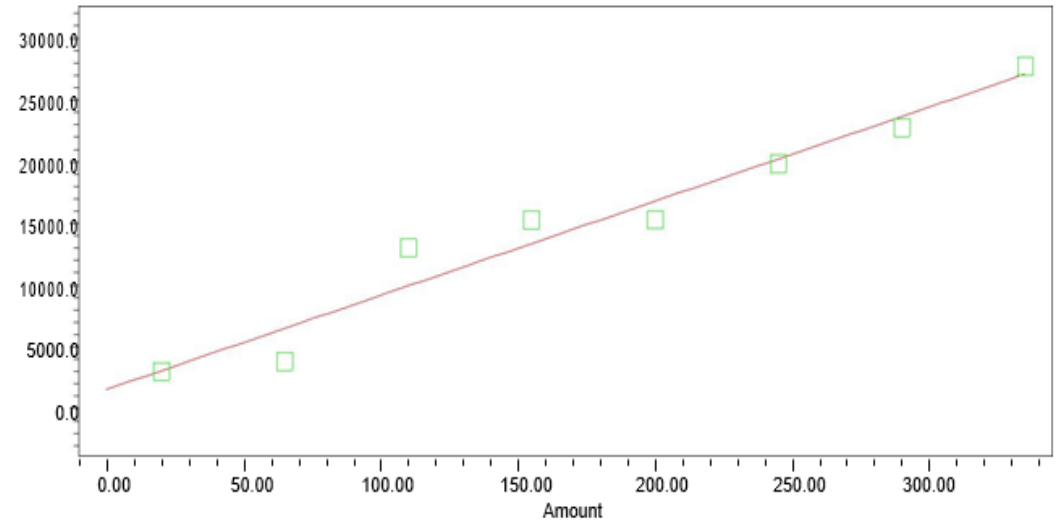


Peak Name: otc; RT: 3.293; Fit Type: Linear (1st Order); Cal Curve Id: 1994; R: 0.965883; R^2: 0.932929; Weighting: None; Equation: Y = 1.11e+002 X - 1.38e+003; Normalized Intercept/Slope: -0.069828; RSD(E): 20.705097

Fig.1: Calibration plot and HPLC chromatogram of standard OTC

	Name	RT	Area	Height	Amount	Units
1		3.194	12053	2300		
2	tc	3.311	27735	2785	335.000	=ppb
3		3.194	12292	2081		
4	tc	3.311	22758	2410	290.000	=ppb
5	tc	3.320	19827	2110	245.000	=ppb
6		3.205	10639	1894		
7	tc	3.197	2996	438	20.000	=ppb
8	tc	3.316	15292	1663	200.000	=ppb
9		3.206	9678	1525		
10	tc	3.316	15267	1647	155.000	=ppb
11	tc	3.197	13023	941	110.000	=ppb
12	tc	3.191	3823	637	65.000	=ppb
13		3.205	9307	1612		

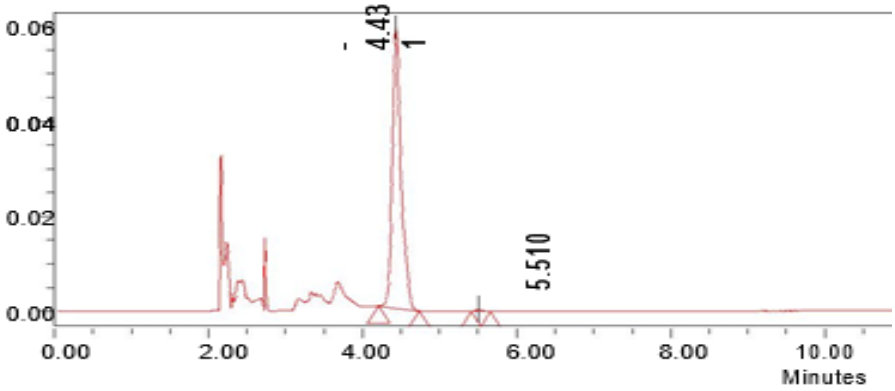




Name: tc; Processing Method: tc calibration final; Fit Type: Linear (1st Order); Cal Curve Id: 1983; A: 1.553054e+003; B: 7.626590e+001; C: 0.000000e+000; D: 0.000000e+000; R^2: 0.953744

Fig.2: Calibration plot and HPLC chromatogram of standard TC

	Name	RT	Area	Height	Amount	Units
1	CTC	4.535	28154	3813	300.000	=ppb
2	CTC	4.505	96522	12879	960.000	=ppb
3	CTC	4.489	155124	20355	1650.000	=ppb
4	CTC	4.475	217522	28697	2310.000	=ppb
5	CTC	4.535				
6		5.510	2633	438		
7		5.523	2129	337		
8	CTC	4.434	421679	59406	4380.000	=ppb
9		5.518	2344	392		
10	CTC	4.431	464064	59162	5070.000	=ppb
11	CTC	4.452	345660	44885	3690.000	=ppb



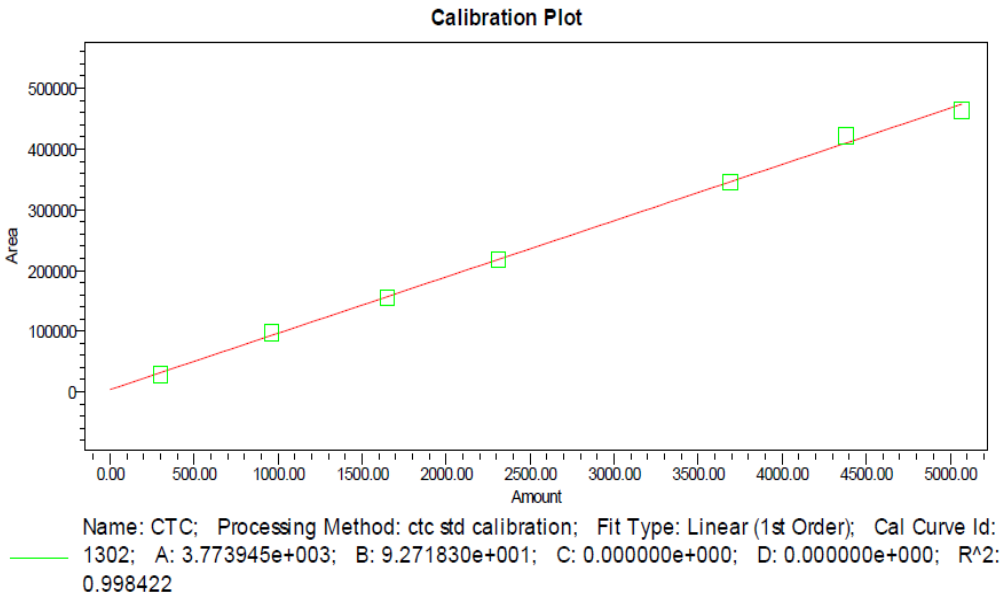


Fig.3: Calibration plot and chromatogram of standard CTC

The retention time of 3.2-3.4 minute for oxytetracycline, 3.311 minute for tetracycline and 4.43 minute for chlortetracycline was obtained whereas the recovery percentage spiked with known concentration of OTC, TC and CTC standards were obtained. Recovery percentage for OTC, TC and CTC in milk sample was 77.38%, 97%, 74% respectively. (Table 2)

Table 2 Retention time, recovery percentage and correction factor of TCs

	Antibiotic	**Retention time (min.)**	**Milk Samples**	
			Recovery (%)	**C.F.**
01	OTC	3.2-3.4	77.38	1.29
02	TC	3.311	97	1.030
03	CTC	4.43	74	1.35

These findings are similar to the reports of Jarunee Loksuwan (2002) who reported 79.89% recovery for OTC, 77.37% recovery for TC and 75.24% recovery for CTC. Similar findings were also reported by Biswas *et al*. (2007) who reported 78% recoveries of all the drugs tested in Buffalo meat in I.V.R.I (U.P) region. Mean recovery for TC (91.5 ± 5.2) were also reported by Fritz and Zuo (2007). However higher rates of recovery percentage were reported by Han *et al*. (2015) who reported 76-116% recoveries of three tetracycline used in the study.

Differences in the recovery percentage of tetracycline residues with the previous studies may be attributed to the multiple factors, such as, type of the column used, method development for single or multi residues, protocol used for

extraction of tetracyclines, slightly higher or lower environmental temperature, column temperature, sample temperature, type of HPLC system used for standardization.

4.2 Detection OTC residues in milk samples

A total of the 200 samples of cow 100 and buffalo 100 milk were analyzed. Out of 200, 11 (cow) and 7 (buffalo) milk samples were found positive for OTC and showed overall prevalence of 9%. District wise highest prevalence (14%) of OTC was detected in Rajnandgaon district followed by Durg (10%), Balod (8%) and Raipur (4%). Highest prevalence of OTC was seen in cow milk (11%) followed by buffalo milk (7%) respectively. (Table 3 and Fig 4).

Analysis of Cow milk sample showed the highest prevalence for OTC in Rajnandgaon (16%) district followed by Durg (12%), Balod (8%) and Raipur (8%), while Buffalo milk samples highest prevalence for OTC in Rajnandgaon (12%) followed by Durg (8%) and Balod (8%), no sample was found positive for OTC residue in Raipur district. (Table 4 and Fig 5-7)

The overall mean residual concentration of OTC was found 0.025±0.007 µg/ml. Maximum concentration of OTC was detected in Durg followed by Rajnandgaon, Raipur and Balod with mean residue levels of 0.047 ± 0.024, 0.029 ± 0.012, 0.016 ± 0.011 and 0.010 ± 0.005 µg/ ml, respectively. The highest mean residual concentration of (0.053±0.035) µg/ml of OTC was found in cow milk sample of Durg district followed by Raipur (0.033±0.022), Rajnandgaon (0.027±0.017) and Balod (0.007±0.006) µg/ml. In buffalo milk samples highest concentration of OTC was observed in Durg district followed by Rajnandgaon and Balod with the mean residual concentration of (0.040±0.035), (0.031±0.019)

and (0.012±0.008) µg/ml respectively. No residue of OTC was recorded from Raipur district in buffalo milk samples. (Table 5-6)

Table 3 District wise positive samples for OTC, TC, CTC residues in milk

S.N.	District	No. of sample analyzed	No. of Positive samples (%)		
			OTC	TC	CTC
1.	Durg	50	5 (10%)	2(4%)	0
2.	Rajnandgaon	50	7(14%)	0	0
3.	Raipur	50	2(4%)	0	0
4.	Balod	50	4(8%)	0	0
	Total	200	18(9%)	2(1%)	0

In the present study out of 200 samples analyzed the prevalence of oxytetracycline residue was observed as 9% which is slightly lower than the recorded values of Gaurav *et al*. (2014) who reported 13.5% prevalence of oxytetracycline residues in cattle milk in Punjab region. On the contrary higher oxytetracycline residue were reported by Manish, (2003) who reported 19.3% prevalence of oxytetracycline residue in meat samples in Pantnagar region. However lower findings were reported by Biswas *et al*. (2007) who reported 4% prevalence of oxytetracycline residue in buffalo meat samples in U.P region.

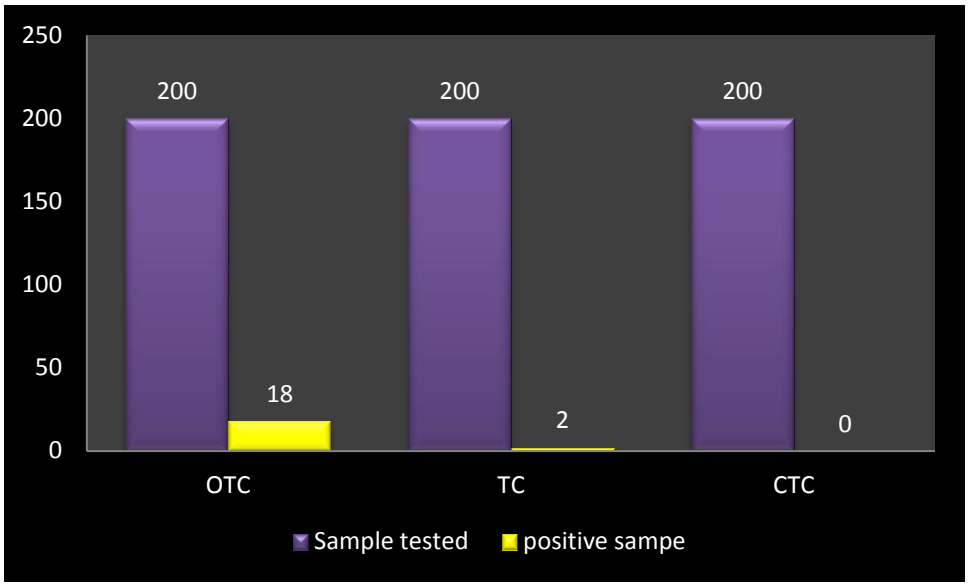


Fig.4: OTC, TC and CTC residues in milk samples

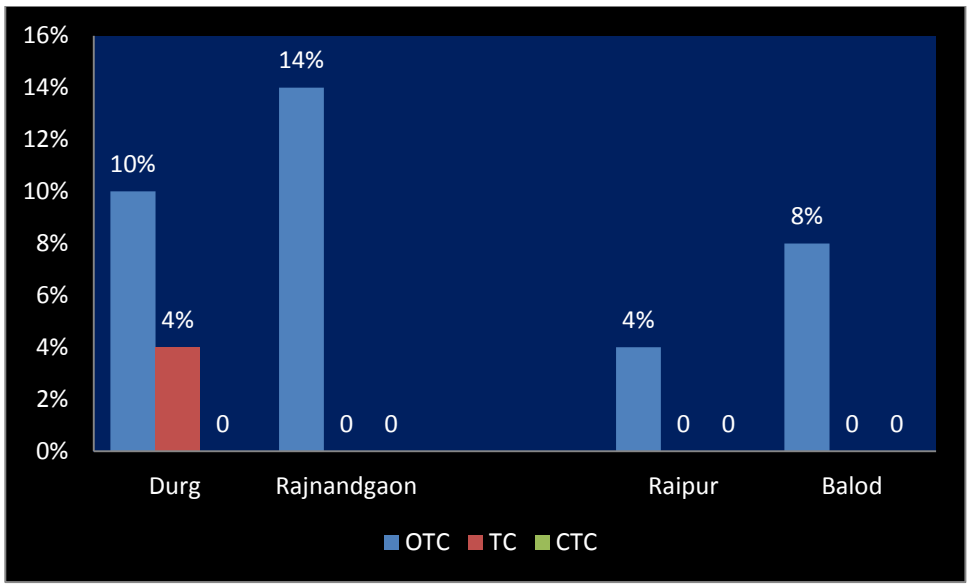


Fig.5: Location wise prevalence of OTC, TC and CTC residues in milk samples

Table 4 Species and different district wise distribution of tetracycline residues in milk

Species	District	Number of samples analyzed	No. of positive samples (%)		
			OTC	TC	CTC
Cow	Durg	25	3(12%)	2(8)	0
	Rajnandgaon	25	4(16%)	0	0
	Raipur	25	2 (8%)	0	0
	Balod	25	2 (8%)	0	0
	Sub Total	100	11 (11%)	2 (2%)	0
Buffalo	Durg	25	2(8%)	0	0
	Rajnandgaon	25	3 (12%)	0	0
	Raipur	25	0	0	0
	Balod	25	2 (8%)	0	0
	Sub Total	100	7 (7%)	0	0
	Total	200	18(9%)	2(1%)	0

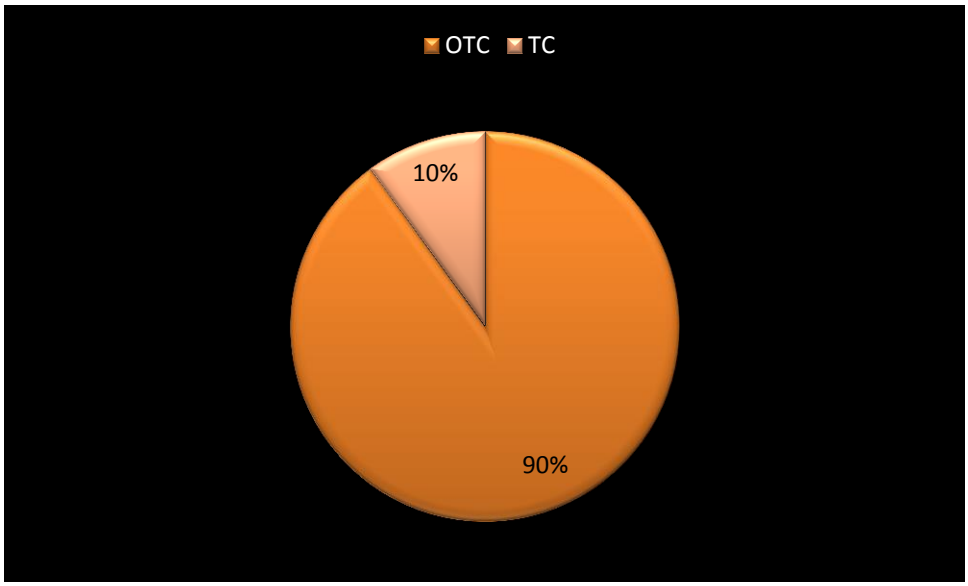


Fig.6: Over all prevalence of OTC, TC and CTC residues in milk samples

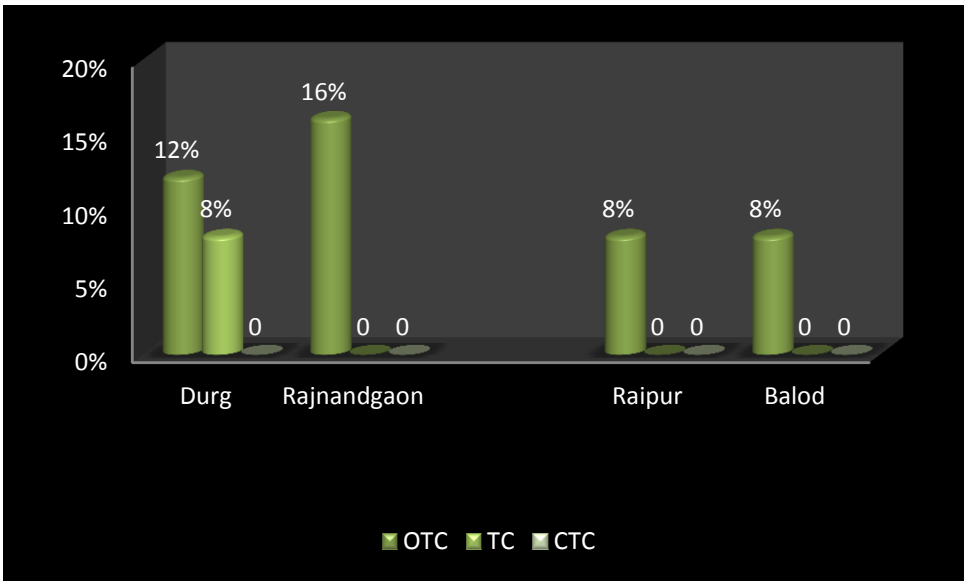


Fig.7: District wise prevalence of OTC, TC and CTC in cow milk samples

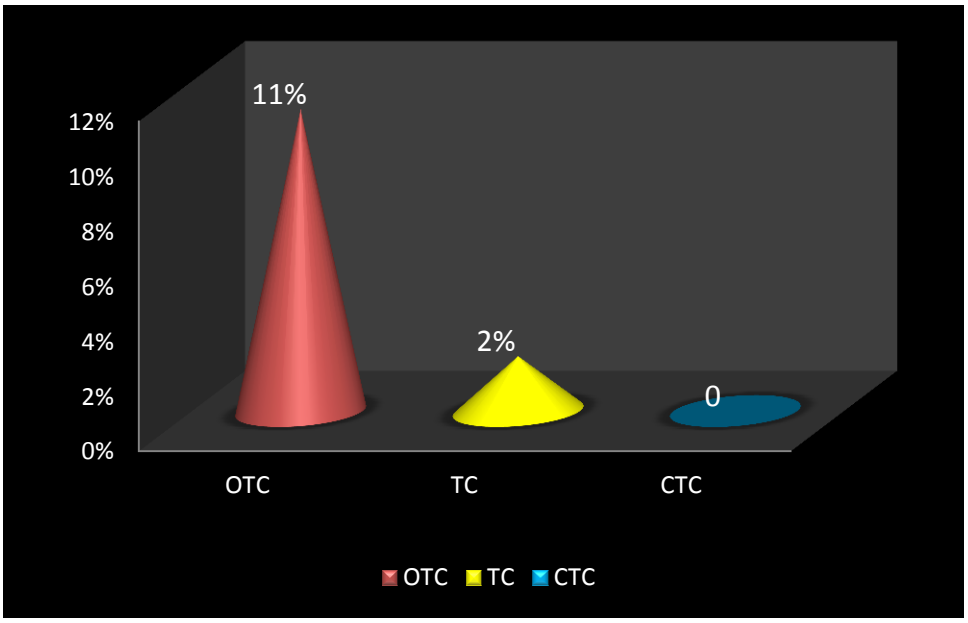


Fig.8: Overall prevalence of OTC, TC and CTC in cow milk samples

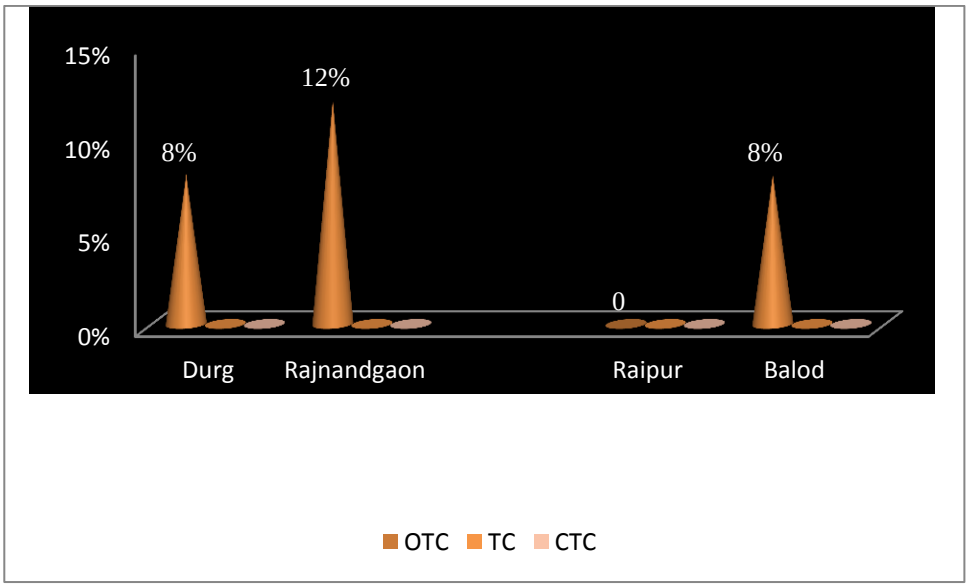


Fig.9: District wise prevalence of OTC, TC and CTC in buffalo milk samples

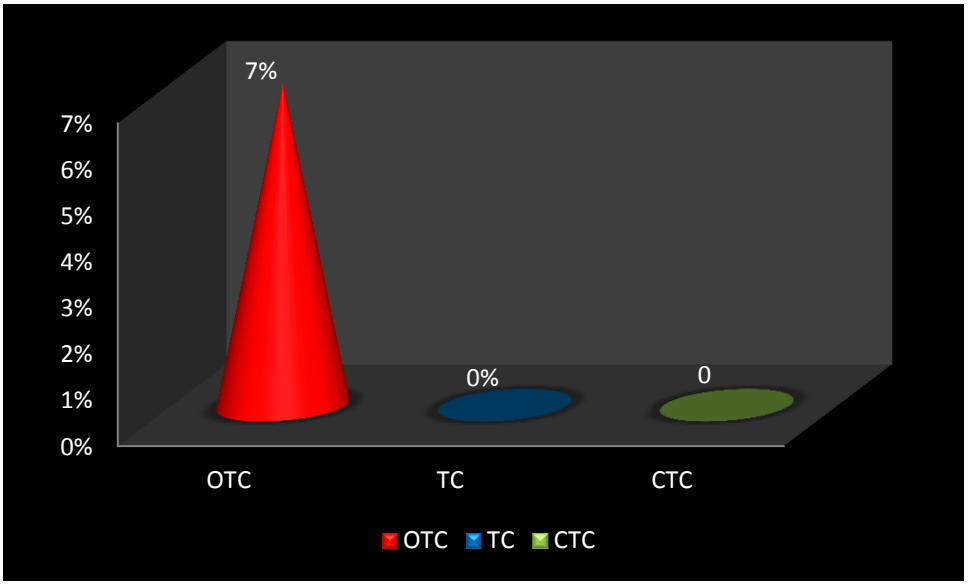


Fig.10: Over all prevalence of OTC, TC and CTC in buffalo milk samples

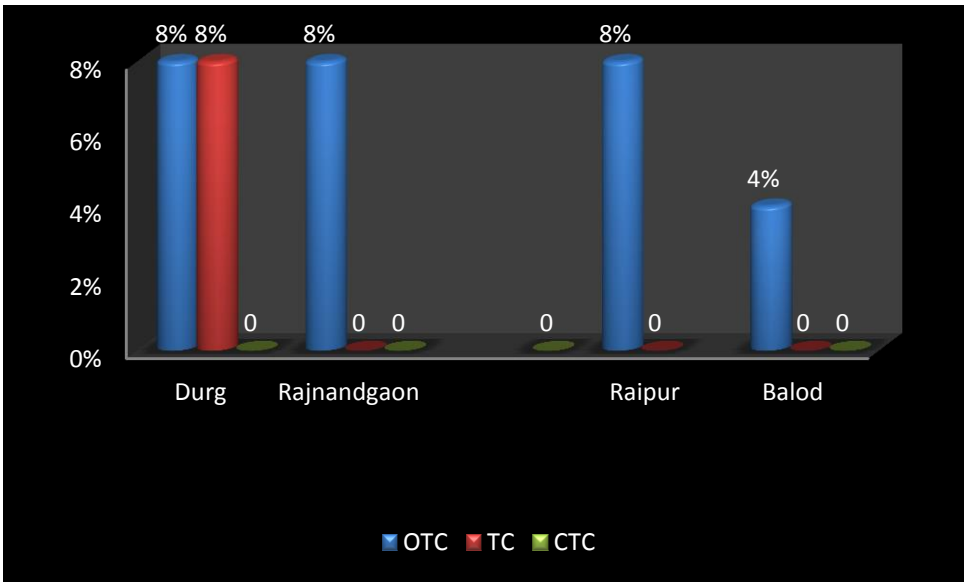


Fig.11: Prevalence of above MRL samples in cow milk

Table : 5 Determination of Mean residual concentration (µg/ml) of OTC, TC and CTC in milk samples

Districts	OTC			TC			CTC		
	Mean ± SE	SD	Range	Mean ± SE	SD	Range	Mean ± SE	SD	Range
Durg	0.047 ± 0.024[a]	0.174	ND -0.870	0.009±0.006[a]	0	0	0	0	0
Rajnandgaon	0.029 ± 0.012[a]	0.091	ND -0.250	0	0.044	ND-0.250	0	0	0
Raipur	0.016 ± 0.011[a]	0.082	ND -0.453	0	0	0	0	0	0
Balod	0.010 ± 0.005[a]	0.038	ND -0.162	0	0	0	0	0	0
Total mean	0.025±0.007[a]	0.108	ND -0.002	0.002±0.001	0.022	0	0	0	0

ND-: Not detected

[a]represent non-significant difference between districts according to one way ANOVA at $p \leq 0.05$ level of significance

Table: 6 Determination of species wise mean residual concentration (µg/ml) of OTC, TC and CTC

Species	Districts	OTC			TC			CTC		
		Mean ± SE	SD	Range	Mean ± SE	SD	Range	Mean ± SE	SD	Range
Cow	Durg	0.053±0.035	0.176	ND-0.71	0.018±0.012	0.062	ND-0.25	ND	ND	ND
	Rajnandgaon	0.027±0.017	0.085	ND-0.36	ND	ND	ND	ND	ND	ND
	Raipur	0.033±0.022	0.114	ND-0.45	ND	ND	ND	ND	ND	ND
	Balod	0.007±0.006	0.032	ND-0.16	ND	ND	ND	ND	ND	ND
Buffalo	Durg	0.040±0.035	0.175	ND-0.87	ND	ND	ND	ND	ND	ND
	Rajnandgaon	0.031±0.019	0.099	ND-0.44	ND	ND	ND	ND	ND	ND
	Raipur	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Balod	0.012±0.008	0.043	ND-0.16	ND	ND	ND	ND	ND	ND

ND-: Not detected

[a] represent non-significant difference between districts according to one way ANOVA at $p \leq 0.05$ level of significance

Table 7 Determination of Mean residual concentration of OTC, TC and CTC in milk samples

Antibiotic	Total Number of samples analyzed	No. of Positive samples	Mean concentration (µg/ml)	MRL (European Union) (µg/ml)	Above MRL positive samples	Above MRL Samples (%)
OTC	200	18	0.025	0.1	14	7%
TC	200	2	0.002	0.1	2	1%
CTC	200	0	0	0.1	0	0

4.3 Determination of tetracycline residue in milk samples

A total of the 200 samples including 100 cows and 100 buffaloes milk samples were screened for the presence of TC residue. Out of 200 samples 2 milk samples were detected positive for TC residue with a overall prevalence of 1%. The highest prevalence rate was found in Durg district (8%). None of the samples were found positive for TC residue in remaining three districts screened. (Figure 4 and Table 4)

Overall prevalence of TC residue found in cow milk was 1% with the highest prevalence of (8%) in Durg. Samples from other district were negative for TC residue. None of the buffalo milk sample were found positive for TC. (Table 4)

The overall mean of 0.002±0.001 µg/ml was detected for TC in the present study. TC residues were detected in cow milk samples of Durg district, with mean level of 0.002±0.001. (Table 5)

In the present study the overall prevalence of TC residue were detected in 2 milk samples (1%) which are similar to the findings of Fritz and Zuo, (2007) who reported the detection of TC residue in only one milk sample. Manish, (2003) reported the overall prevalence of 7.16% TC residue in meat samples of Pantnagar region which was higher than the 1% TC residue detected in the present study. The mean residual concentration of tetracycline was 0.009±0.006 µg/ml found in the present study which is higher than the recorded residual values of Tona and Olusola who reported 0.003±0.001 µg/ml mean residual concentration of TC in Nigeria.

4.4 Determination of chlortetracycline residue in milk samples

A total of 200 (100 cows and 100 buffaloes) were screened in the present study, no samples was found positive for chlortetracycline residue. This finding of the present study is similar to the findings of Biswas *et al*. (2007) who screened 122 buffalo meat samples for CTC and found none for the presence of CTC in U.P.

4.5 Comparison of results found in the present study with the MRLs prescribed by European Union

European Union had given maximum residue limit of 0.1 µg/ml for individual tetracycline and combined maximum residue limit of 0.3 µg/ml established by the US Food and Drug Administration. Out of total milk samples analyzed, 14 (7 cow and 7 buffalo) milk samples violated the MRL value prescribed by European Union for OTC residue. Both cow and buffalo milk samples shown an overall prevalence rate of 7%. District wise prevalence for cow milk samples was 8% (2 sample) for Durg district followed by 8% (2 sample), 8%

(2 sample), 4% (1 sample) from Rajnandgaon, Raipur and Balod district respectively and violated the MRL value. Maximun concentration of 0.714 µg/ml OTC residue was detected in Durg District and minimum concentration of 0.035 µg/ml was recorded in Balod district. One milk sample from Durg, two from Rajnandgaon and one milk sample from Balod district was found below the MRL value. Whereas in buffalo milk samples, district wise highest prevalence was reported in Rajnandgaon district where 3(12%) milk samples followed by 2(8%) from Durg and 2(8%) milk samples from Balod violated the MRL value. All 7 buffalo milk samples violated the MRL value prescribed by European Union. Maximum concentration was recorded in Durg district i.e. 0.870 µg/ml and minimum concentration was recorded in Rajnandgaon district i.e. 0.113 µg/ml. Out of total samples screened only 2 cow milk samples having TC residue were recorded in Rajnandgaon district and violated the MRL value prescribed by European Union. The maximum concentration was 0.250 µg/ml and minimum concentration recorded was 0.200 µg/ml. In the present study no samples for chlortetracycline residue were found positive. (Table 8)

Significantly higher level of oxytetracycline residues were recorded by Kimera *et al*. (2015) who reported 68.3% of total oxytetracycline residue above maximum residue limits in meat samples in the Kilosa district, Tanzania as compared to 7% found in the present study.

Table: 8 Location and species wise milk samples above MRL values

Species	Districts	Number of samples Analyzed	OTC		TC		CTC	
			Positive Samples (%)	European Union MRL Above samples (%)	Positive Samples (%)	European Union MRL Above samples (%)	Positive Samples (%)	Codex MRL Above samples (%)
Cow	Durg	25	3 (12%)	2 (8%)	2(8%)	2(8%)	0	0
	Rajnandgaon	25	4 (16%)	2(8%)	0	0	0	0
	Raipur	25	2 (8%)	2(8%)	0	0	0	0
	Balod	25	2(8%)	1(4%)	0	0	0	0
	Sub Total	100	11(11%)	7(7%)	2(2%)	2 (2%)	0	0
Buffalo	Durg	25	2(8%)	2(8%)	0	0	0	0
	Rajnandgaon	25	3(12%)	3(12%)	0	0	0	0
	Raipur	25	0	0	0	0	0	0
	Balod	25	2(8%)	2(8%)	0	0	0	0
	Sub Total	100	7(7%)	7(7%)	0	0	0	0
	Grand Total	200	18(9%)	14(7%)	2(1%)	2 (1%)	0	0

In the present study oxytetracycline was found to be the major antibiotic used which is widely used as broad spectrum antibiotic for the prevention and treatment of great number of diseases. Low level of antibiotic residue if consumed for a long period of time can lead to the development of drug-resistant microorganisms. Oxytetracyclines are widely used to treat dairy cattle diseases such as mastitis, diarrhea and pulmonary diseases, or to increase milk yield. Apart from being a broad spectrum antibiotic, oxytetracycline is also cheap, readily available from veterinary shops and accessed easily, without restrictions. Fourteen milk samples of oxytetracycline residue violated the MRL set by Eueopean Union. This is probably due to the milk samples collected during the antibiotic treatment of animal, milk samples collected before the completion of withdrawal period and overdose of oxytetracycline drug used. So there are chances of this compound to be present in milk above MRL.

In the present study out of total samples analyzed only two milk samples were found positive for tetracycline residue which is not surprising because tetracycline undergoes epimerization to form 4-epitetracycline in animal stomach fluids. The other possible reason may be that this particular drug is not used by the farmers for therapy purpose.

In the present study chlortetracycline residue were detected in none of the samples because of its unavailability from the market and today this drug is widely used for treatment in the poultry industry only, it is not prescribed by any veterinarian and nor used by the farmers anymore for the treatment purpose.

4.6 Exposure assessment of tetracycline residues in milk

Estimation of risk assessment based on hazard quotient

The ingestion of residues of antimicrobial compounds in food of animal origin may also pose a danger to human health by colonization, barrier disruption leading to pathogenic bacteria overgrowth or by exerting a selective pressure on the intestinal micro flora thus favoring the growth of microorganisms with intrinsic or acquired resistance. The toxicological or microbiological endpoint resulting in lowest ADI ultimately drives the overall ADI. Thus, the ADI is determined as conservative estimate of safe ingestion levels by humans based on the lowest ADI among the toxicology and microbiology studies.

The estimated daily intake was calculated by using following equation as given by (Jaun *et al*., 2010)

$$EDI = \frac{\text{(Mean of mg antibiotic per kg of food)} \times \text{(Daily intake of food)}}{\text{Adult body weight 60 kg}}$$

and,

Hazard Quotient = EDI / ADI.

The per capita availability of milk in Chhattisgarh state was estimated to be 130 gm/day (Government of India, 2014).

In the present study, mean level of residue of oxytetracycline and tetracycline in raw milk samples were found to be 25µg/kg and 2µg/kg, respectively. Based on the mean value of antibiotic residues hazard quotient were calculated for oxytetracycline and tetracycline residues as 0.0018 and 0.0001, respectively. (Table 9)

Table: 9 Estimation of risk assessment based on Hazard Quotient for tetracycline residue (mean concentration)

Antibiotic	EDI (µg/kg/day)	ADI (µg/kg/day)	Hazard quotient	References
Oxytetracycline	0.054	30	0.0018	JECFA, 2002
Tetracycline	0.004	30	0.0001	JECFA, 2002; OCS, 2013

Elizabeta *et al*. (2011) calculated the estimated daily intakes (EDI) for the average daily consumption of 200 ml of milk in Macedonia, for the examined antimicrobials and obtained level of 2 to 100 times lower than the values of acceptable daily intake fixed by World Health Organization which is higher than the result obtained in the present study which is 500 times lower as compared to JECFA standards.

In the present study, the values obtained were 500 times lower than the values of acceptable daily intake but higher than the report of Bilandzic *et al* (2011) who reported 20 to 1620 times lower values than acceptable daily intake fixed by World Health Organization.

The result obtained in the present study indicated that toxicological risks associated with the consumption of analyzed milk could not be considered as public health issue with regards to these veterinary drugs.

CHAPTER - V

SUMMARY, CONCLUIONS AND SUGGESTIONS FOR FUTURE RESEARCH WORK

Milk is an important inexpensive dietary source which contains valuable proteins and calcium essential for promoting growth in children and general health of population. The presence of antibiotic residues in foods has attracted considerable attention in recent times. Because of their wide application, there is a concern about the presence of residues in milk which may result from any of the following: excessive use, improper use, or a shortened withdrawal time. The presence of such residues in foods not only poses a direct threat to the health of the consumers but also has industrial and other implications. Main problem associated with the increase of intestinal flora, increase of the selection pressure and spread of drug resistant bacteria within animal and human populations. The present study was planned to screen the milk samples collected from four districts, Durg, Balod, Raipur and Rajnandgaon of Chhattisgarh, for the presence of antibiotic residues in cow and buffalo milk samples. The procedure for extraction, detection and quantification of tetracycline residues was standardized. The retention time of OTC, TC and CTC was calculated with the help of respective standards. Blank milk samples were spiked with known concentration of antibiotics to calculate the recovery percentage. Further correction factor was also calculated by using recovery percentage.

Mcllvaine-EDTA-NaCl buffer was used as a extracting solvent which was used during sample extraction procedure, detection and quantification of antibiotic residues was done by using High performance liquid chromatography (HPLC)

with photo diode array detector. Oxytetracycline, tetracycline and chlortetracycline was eluted through C18 column with retention time of 3.2-3.4, 3.31 and 4.43 min respectively. Different concentrations of OTC, TC and CTC standards were made and standard calibration curves were generated by plotting peak areas against their respective concentrations.

A total of 200 samples (100 cow and 100 buffalo) were screened for the presence of OTC, TC and CTC residues. The results obtained after screening 200 samples revealed that 18 (9%) samples showed the presence of OTC and 2(1%) samples were found positive for TC residues whereas not a single sample was found positive for chlortetracycline residues. A total of 7(7%) cow and 7(7%) buffalo milk samples containing OTC and TC respectively were found to be violating the MRL value.

During the entire period of study residues of OTC were detected in 11(11%) cow and 7(7%) buffalo milk samples. Similarly, TC residues were detected in 2(1%) cow milk samples. District wise highest prevalence were recorded in Rajnandgaon (14%) followed by Durg (10%), Balod (8%) and Raipur (4%) in cow milk samples. District wise highest mean residual concentration was recorded in Durg (0.053±0.035 µg/ml) followed by Raipur (0.033±0.022 µg/ml), Rajnandgaon (0.027±0.017 µg/ml) and Balod (0.007±0.006 µg/ml).

The result obtained in the present study indicated that OTC was probably used more widely in therapy as compared to TC. Indiscriminate use of antibiotics in food animals, administration of drugs in unacceptable dosages for an inappropriate period and through abnormal routes generally leads to the presence

of residues in food. However, out of 20 samples found positive for both OTC and TC residues, 16 samples were found to violate the MRL value, so there is need of monitoring and surveillance programmes in foods, enhancing consumer awareness about the ill effects of residues in foods.

CONCLUSIONS

1. The overall rate of prevalence of OTC and TC in milk samples was 9% and 1% respectively whereas and no case of CTC was recorded.
2. In cow milk sample the prevalence of OTC and TC was 11% and 1% respectively.
3. In Buffalo milk samples the recorded prevalence of OTC was 7%.
4. The mean residual concentration of OTC was 0.025 µg/ml and TC was 0.002.
5. The frequent use of oxytetracycline in mastitis control programme and due to its broad spectrum activity may be the possible reason for the contamination of milk by this antibiotic. Since only 2 samples were found positive for tetracycline possibly because this antibiotic nowadays not generally used for therapy purpose.
6. No residue of chlortetracycline was found in any milk sample may be due to its unavailability in market. Chlortetracycline nowadays widely used for treatment in poultry industry.
7. There is a need to educate the consumers and dairy farmers emphasizing the public health importance of haphazard use of antibiotics and their withdrawal time.

SUGGESTIONS FOR FUTURE RESEARCH WORK

1. A comprehensive study in human and different species of animals with more number of samples from different districts can be done for ascertaining the status of antibiotics in Chhattisgarh
2. Microbial screening for the presence of antibiotics can be carried out by using different kits available in the markets.
3. LC-MS/MS quantification of antibiotics can be carried out.
4. Pre heat treatment of milk can be carried out to check the status of recovery percentage after heating of different antibiotics.
5. Antibiotic residues in meat samples of birds and animals can be done.

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Appendix

PERFORMA FOR COLLECTION OF SAMPLES FROM AN ANIMAL FARM CHHATTISGARH KAMDHENU VISHWAVIDHYALAYA, DURG (C.G.)

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND EPIDEMIOLOGY

1.Name of the dairy Farm/Owner
2.CompleteAddress
3.Identification of animal Cow Buffalo ☐ ☐
4. Any treatment given to animal
5. Remark

The maximum residue limits (MRLs) for different pesticides in milk

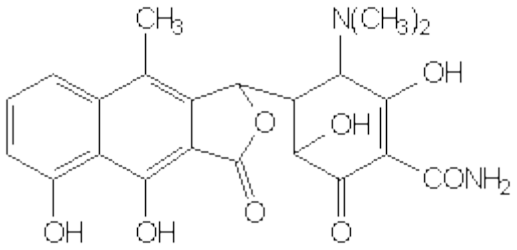
Antibiotics	**MRL level of Antibiotic in milk as per European Union**
OTC	100ppb
TC	100ppb
CTC	100ppb

Chemical properties of tetracyclines

The reactions that tetracyclines undergo are generally of a sophisticated nature, dictated by the complex functionality and the sensitivity of the molecules to mild reaction conditions (acid, base, heat)

Acidic conditions

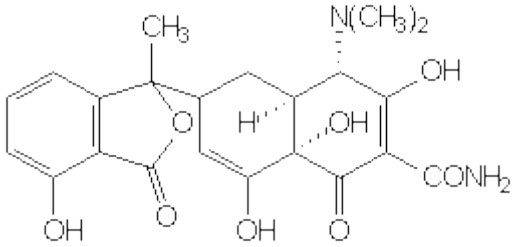
When exposed to dilute acid conditions, tetracycline undergoes dehydration to yield anhydrotetracycline. Anhydroterramycin suffers further cleavage and lactonization to apoterramycin:



Diluted acid promotes epimerization at C-4 as well.

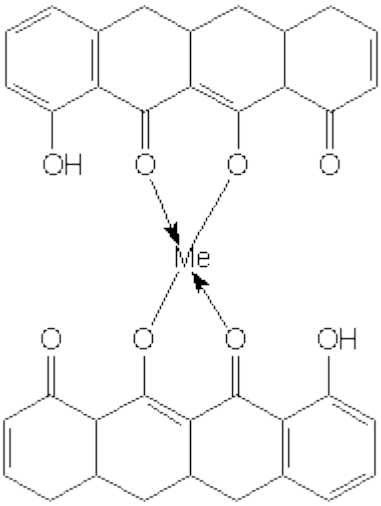
Basic conditions

Mild alkali attacks 11a carbon of tetracycline, which is transformed to isotetracycline:



- **Formation of complexes**

Tetracycline possesses a great tendency to form complexes with a number of chemical species, due to its B- and C-ring oxygen atoms:



It complexes most readily with Fe^{3+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Be^{2+}, Al^{3+} among metal ions, phosphates, citrates, salicylates, *p*-hydroxybenzoates, saccharin anion, caffiene, urea, thiourea, polivinylpyrrolidone, serum albumin, lipoproteins, globulins, and RNA .

THESIS ABSTRACT

Title of Thesis: Quantitative analysis of tetracycline residues in cow and buffalo milk in Chhattisgarh

Name of Student: Praveen Kumar (Roll No. 201404009, ID No- K10106021)

Tetracyclines are the group of broad-spectrum antibiotics that have been used for more than 50 years for the treatment of bacterial infections in both humans and animals. The present study was undertaken to optimize the high performance liquid chromatography protocol for the detection of antibiotic residues in milk, quantitative analysis of tetracycline residues in cow and buffalo milk samples and to estimate the exposure assessment of tetracycline residues in milk. A total of 200 milk samples (100 cow and 100 buffalo milk samples) were screened for the presence of oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) residues.

Milk samples were collected from cows and buffaloes from Raipur, Balod, Rajnandgaon and Durg districts of Chhattisgarh. The procedure for the extraction, detection and quantification of OTC, TC and CTC was standardized. Sample extraction was done by adding 2 ml of Mcllvaine-EDTA-NaCl buffer in 2 ml of test milk sample then homogenized in vortex for 10 minute, centrifuged at 8000 rpm for 30 min at 10^0C and supernatant were collected and filtered through 0.22 µm PVDF membrane and finally 100 µl of aliquot were injected in HPLC system for analysis. To quantify the tetracycline residues in milk, C18 column was used. The mobile phase of (a) acetonitrile (b) methanol: acetonitrile: 0.01 M oxalic acid (1:1.5:4.5) composition was used to elute the tetracyclines followed by gradient

programme, wavelength of 375 nm with a flow rate of 1ml/min was used. The retention time of OTC, TC and CTC were 3.2, 3.311 and 4.43 minute respectively, the recovery percentage of OTC, TC and CTC were 77.38, 97 and 74 percent and correction factor obtained were 1.29, 1.030 and 1.35% for OTC, TC and CTC respectively.

Out of 200 samples, 18 (9%) and 2(1%) samples were found positive for OTC and TC residues, respectively. The OTC residues were detected in the 11 (11%) cows and 7 (7%) buffaloes milk samples. The OTC highest mean residual concentration of 0.302 µg/ml was detected in buffalo milk followed by 0.278 µg/ml in cow milk samples. A total of 14 milk samples, 7 cow and 7 buffalo milk samples, were found positive for OTC above maximum residual level value. The TC residue was detected in two milk samples with concentration above MRL value. In Rajnandgaon district maximum number of 7 out of 25 milk samples were found positive for OTC above MRL values as compared to minimum number of 2 out of 25 milk samples in Raipur district. None of the samples were found positive for CTC residues. The result obtained in the present study indicated that OTC is widely used as therapy as compared to TC, thus there is a need for monitoring the level of residues in various foods so as to minimize the exposure risk to public health.

Dr. Sanjay Shakya

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