“DETECTION OF ANTIBIOTICS RESIDUES IN MUSCLE, LIVER AND KIDNEY OF CHICKEN”

THESIS

Submitted to the

Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya, Jabalpur

In partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

In

PHARMACOLOGY AND TOXICOLOGY

By

MAHESH S. TRIVEDI

Department of Pharmacology and Toxicology
College of Veterinary Science and Animal Husbandry
Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya, Jabalpur (MP)

2012

1. INTRODUCTION

Antibiotics are substances that kill or inhibit the growth of bacteria. They are one of the most important bioactive and chemotherapeutic groups of compounds made by microbiological synthesis (Joshi, 2002). Today, many antibiotics are widely used for preventing and treating several diseases, as well as for promoting growth in food-producing animals (Di Corcia and Nazzari, 2002). This widespread use of antibiotics, the failure to follow label directions or inappropriate withdrawal period of time before slaughtering of animals may cause residuals in foodstuffs of animal origin.

Many of the antibiotics used to treat bacterial infections in humans have veterinary applications; prophylactics and growth promoters. In these two cases, the antibiotics are used at concentrations lower than therapeutic concentration for a longer period of time; a potentially dangerous practice since it is one of the strongest selective pressures leading to emergence of antibiotic resistant strains of bacteria (Simonsen *et al.,* 1998), induction of allergic reactions in humans and technological problems of fermented meat products (Pavlov *et al.,* 2005). Some antibiotics are directly toxic, like chloramphenicol which causes aplastic anemia, while allergic reactions and toxic side effects may have fatal consequences (Popelka *et al.,* 2003). Therefore protection of public health against possible harmful effects of antibiotic residues is a major concern.

Screening of food products from animal origin for the presence of antibiotics residues started soon after the introduction of antibacterial therapy in veterinary practice. The availability of simple and reliable screening systems for the detection of antibiotics is an essential tool in assuring the safety of food products. Microbial inhibitions assays were the earliest methods used for the detection of antibiotic residues (Mitchell *et al.,* 1998) and they are still widely used. In many countries, governmental authorities have established monitoring programmes to determine antibiotic levels in foods, as well as the highest allowable residue levels (Ramrrez *et al.,* 2003). Foodstuffs containing levels of antibiotics that exceed the tolerance levels must be verified by highly selective and sufficiently sensitive chemical methods (Caren

et al., 2001). High performance thin layer chromatography is a sensitive and reliable method for the qualitative and quantitative detection of antibiotic residues in meat.

Jabalpur district, known as “SANSKARDHANI” is a city in the Mahakaushal region of state of Madhya Pradesh in India. It is the largest urban agglomeration and the third most populous city in Madhya Pradesh. Jabalpur has an extensive network of broiler chicken poultry farms throughout the district. All in all, more then 250 broiler chicken poultry farms including both organized and unorganized are located in and around Jabalpur. Another vital aspect of poultry industry is the supply of chicken meat for human consumption which has been estimated approximately to the extent of more then 15 ton per day.

The present study is therefore aimed, to determine the antibiotics residues in muscle, liver and kidney samples of chicken, using both a microbial growth inhibition assay and high performance thin layer chromatography. The microbial assay was employed to prescreen the possible antibiotic-containing meat samples. The study will also provide further authentic information on use of various antibiotics and their residues in muscle, liver and kidney samples of chickens available in Jabalpur.

OBJECTIVES:

1. To conduct surveillance on use of antibiotics in chicken poultry farms in Jabalpur.
2. To screen chicken samples for presence of antibiotic residues on the basis of microbial growth inhibition assay.
3. To determine residual concentration of antibiotics in muscle, liver and kidney of chickens by using High Performance Thin Layer Chromatography (HPTLC).
4. To determine the effect of boiling on antibiotic residue in edible tissues of chicken.

2. REVIEW OF LITERATURE

Since the 1950s antibiotics have been widely used in food animal production. Food animals are raised in groups or herds, often in confined conditions that promote the spread of infectious diseases. Antibiotics are frequently used to compensate for poor production practices. Most of the antibiotics used in food animals are the same as or belong to the same classes as those used in humans. Antimicrobial classes used to treat chickens include quinolones, tetracyclines, beta-lactams, aminoglycosides, macrolides, polypeptides and sulphonamides (Stolker and Brinkman, 2005).

Antibiotics are used largely for three 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 routinely used at sub-therapeutic levels as animal feed additives (Okerman *et al.,* 1998). The growth promoting effect of antibiotics was discovered in the 1940s, when it was observed that animals fed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues improved their growth. The mechanism of action of antimicrobial agent as growth promoters is related to interactions with intestinal microbial population (Dibner and Richards, 2005; Niewold, 2007).

The term “Antimicrobial Growth Promoter” (AGP) is used to describe any medicine that destroys or inhibit bacteria and is administered at a low, sub therapeutic dose for the purpose of performance enhancement. The use of antimicrobials for growth promotion has arisen with the intensification of livestock farming. Antimicrobial growth promoters are used to “help the animals to digest their food more efficiently, get maximum benefit from it and allow them to develop in to strong and healthy individuals”. As prevention of diseases, enhancement of growth and feed efficacy are crucial to vital animal husbandry business, the use of AGP is increasing day-by-day (Ellin Doyle, 2001).

The specific physiological basis of the growth promoting effects of antibiotics is unknown, but is hypothesized to involve a nutrient sparing

effect in the gut and selective suppression of species of bacteria and clinical expression of infection, i.e., disease prophylaxis (McEwen and Fedorka-Cray, 2002). AGPs are typically administered in sub-therapeutic doses for long periods of time (usually greater than 2 weeks), and sometimes for the entire duration of the production cycle. It was thought that AGPs improved production by 2-10%.

Existence of antibiotic residues in food stuff can pose hazards to human health. Among them are sensitivity to antibiotics, allergic reactions and imbalance of intestinal microflora, bacterial resistance to antibiotics in microorganisms and losses in the food industry (Cunha, 2001; Kirbis, 2006; Lolo *et al*., 2006). Also, the high level of use in animals and humans, unnecessary use or use of quinolones with poor activity in developing countries like India and Iran has been blamed for the rapid development of bacterial resistance to these agents and it has not remarked to withdrawal times of antibiotics in food-producing animal industry. So, quality control of food stuff regarding to antibiotic residues, is necessary (Salehzadeh *et al.*, 2007).

2.1 ANTIBIOTIC RESIDUES IN CHICKEN MEAT:

The world-wide commercial poultry industry is well-developed and is the largest supplier of animal protein in the form of meat and eggs. Its significance is even greater in developing countries, usually providing both protein and income for small family (Law and Payne, 1996). In poultry, antibiotic usage had facilitated their efficient production and also enhanced the health and well-being of poultry by reducing the incidences of disease, but unfortunately, Unauthorized use of these antibiotics, the failure to follow label directions or inappropriate withdrawal period of time before slaughtering of animals could lead to contamination of edible poultry tissue with antibiotic residues, with potential adverse effects on human health (Donoghue, 2003). Antibiotic residues in foods of animal origin are one of the sources of concern among the public and medical health professionals (Adams, 2001).

Assessment of antimicrobial usage and antimicrobial residues in broiler chickens in Morogoro Municipality was done by Nonga *et al.* (2009).

Seventy broiler chicken liver samples were collected for quantitative antimicrobial residue analysis by use of two parallel tests; agar well diffusion and Delvotest SP® assay. The results indicated that 70% of the farms are positive for antimicrobial residues. Ninety percent of the respondents had knowledge on antimicrobial withdrawal period. However, 95% of farmers slaughtered their chicken before withdrawal period because they were afraid of losses and unaware of the effects of antimicrobial residues in humans. They suggest that poultry farmers need to be educated on the possible effects associated with use of food with antimicrobial residues.

Salehzadeh *et al.* (2007) collected 270 chicken muscle, liver and kidney samples from 90 broiler farm in Tehran province. HPLC was used for separating detecting and analyzing of enrofloxacin residues in samples. Sample from 22 (24%) of farms show residue above Maximum Residual limits (MRLs). The enrofloxacin positive samples, which showed residues of enrofloxacin above MRLs, were 8 (8.88%), 12 (13.33%) and 22 (24.44%) of muscle, liver and kidney samples showed residue of enrofloxacin above MRLs respectively. The mean concentrations of enrofloxacin in muscle, liver and kidney samples were 18.32±32.29, 18.34±12.36 and 26.06±19.52 ng/g respectively.

Shahid *et al.,* (2007) conducted a study to monitor the presence of antibiotic residue in poultry meat. Swab test on food animal employing *Bacillus subtilis* as test organism on nutrient agar was used. A total of 100 tissue samples (33 liver, 33 kidney and 34 muscles) were collected from local market of Rawalpindi and Islamabad. Thirteen liver (39.4%), 9 (27.3%) kidney and 7 (20.6%) muscle sample were detected positive for antibiotic residues.

Petrovic *et al.* (2006) studied the presence of enrofloxacin residues in muscle and liver after peroral administration of enrofloxacin (10 mg/kg b.wt./day) to chickens. Microbiological method – plate pH 8.0 with Escherichia coli NCIMB 11595 and HPLC with fluorescence detection were used for the detection of enrofloxacin residues. During the 5 days dosing period, enrofloxacin concentrations in breast muscle and liver greatly exceeded the EU MRL values. Ciprofloxacin was not detected in muscles

after 2 days of dosing, but it was detected in the liver in concentrations above the MRL. During the post treatment period, enrofloxacin and ciprofloxacin concentrations in the breast muscle and liver exceeded the MRL values until the 4th day of the withdrawal period. Ciprofloxacin was not detected in the breast muscle after 4 days post dosing, but it was detected in the liver at concentrations below MRL. Four days withdrawal period is the allowed time for the drug concentration to decrease to an acceptable level in the meat and liver, prior to slaughter (below MRL).

Pavlov *et al.* (2005) studies the residue levels of tobramycin in muscle (115), liver (192) and kidney (155) samples of poultry stored at -18^{o}C. The residues of tobramycin were determined over a period of 60 days using microbiological method. They found a decreasing level of tobramycin during period of storage. They found that 4% muscle, 17% liver and 33% kidney sample were positive for antibiotic residues.

Alhendi *et al*. (2000) studied drug residues in broiler chickens fed with antibiotics in ration. Daily oral administration of two dose levels of 1 and 2 mg/kg body mass of ampicillin (groups A1 and A2), 50 and 100 mg/kg body weight of oxytetracycline (groups O1 and O2) and 50 and 100 mg/kg body mass sulphadimidine (groups S1 and S2), in broiler feed resulted in an immediate increase in concentrations of antibiotics in plasma and tissues from day 1 until day 40 of the treatment. At day 40 a range of 0.61 to 1.94, 0.24 to 2.25, 1.30 to 6.70 µg/g or µg/ml of A, O and S, respectively was found in tissues or plasma. Withdrawal of medicated feed resulted in a rapid decline in tissue concentration parallel to that of plasma, and withdrawal times were 5 days for oxytetracycline and sulphadimidine and 6 days for ampicillin.

Al-Ghamdi *et al.* (2000) collected chicken muscle, liver and egg samples from 33 broiler and 5 layer farms in the eastern province of Saudi Arabia. Antibiotic-residue positive samples were identified in the products of 23 (69.7%) broiler and 3 (60%) layer poultry farms. All the antibiotic-residue positive broiler farms were positive for at least one tetracycline compound in raw muscle (87%) and liver (100%) respectively, while 73.9% broiler farms were positive for 2 or more tetracyclines in these two tissues. Furthermore, 82.6% of the antibiotic-residue-positive farms had mean concentrations of at

least one tetracycline compound in excess of the permissible maximum residue limit (MRL) in raw muscle and liver. This study confirmed widespread misuse of tetracycline agents including multiple uses of drugs belonging to the same pharmacological group and lack of implementation of recommended withdrawal times.

2.2 TOXICITY OF ANTIBIOTIC RESIDUES:

The residues of antibiotics or its metabolites in meat and other foods of animal origin may cause adverse effects on consumers' health. The presence of residues and its associated harmful health effects on humans makes the control of antibiotics residues an important measure in ensuring consumer protection. Other important effects mainly due to the presence of residual antibiotics consist in allergic reactions or the selection of resistant bacteria that could be transferred to humans through the food chain (Butaye *et al.,* 2001). In addition, the consumption of trace levels of antibiotics residues in foods from animal origin may have consequences on the indigenous intestinal microflora which constitutes an essential component of human physiology. This flora acts as a barrier against colonization of the gastrointestinal tract by pathogenic bacteria (Vollard and Clasener, 1994) and has an important role for food digestion. So, the ingestion of trace levels of antimicrobials in foods must take into account potentially harmful effects on the human gut flora (Cerniglia and Kotarski, 1999).

A number of possible adverse health effects of antibiotics residues have been suggested. These include the following:

- Allergic/Anaphylactic reactions to residues.
- Chronic toxic effects occurring with prolonged exposure to low levels of antibiotics.
- Development of antibiotic-resistant bacteria in treated animals. These bacteria might then cause difficult-to-treat human infections.
- Disruption of normal human flora in the intestine. Antibiotics might reduce total numbers of these bacteria or selectively kill some important species.

2.21 Allergic/Anaphylactic reactions to residues

A few reports indicate that sensitive individuals may experience allergic reactions to antibiotics residues, particularly penicillin residues, in meat. Anaphylactic reactions have been reported to result from consumption of meat containing penicillin (Gomes and Demoly, 2005; Raison-Peyron *et al.,* 2001). It is possible that some minor reactions, such as skin rashes, may also have occurred but these have not been reported. Estimates of the prevalence of drug sensitivity vary but are estimated to be about 7% in the general population. However, not all of these people experience severe symptoms, and residue levels detected in meat are likely to be below the threshold that would induce a hypersensitive response (Paturkar *et al.,* 2005; Waltner-Toews and McEwen, 1994).

Tetracycline agents are of great clinical importance because they possess a wide range of antimicrobial activity against aerobic and anaerobic gram-positive and gramnegative bacteria. They are also effective against some microorganisms that are resistant to cell-wall-inhibitor antimicrobial agents, such as Rickettsia, Mycoplasma pneumoniae, Chalamydia spp., Legionella spp., Ureaplasma, some atypical mycobacteria and Plasmodium spp.

Symptoms of chronic exposure to oxytetracycline (OTC) include blood changes such as leucocytosis, atypical lymphocytes, lung congestion, toxic granulation of granulocytes and thrombocytopenia purpura. Liver injury and delayed blood coagulation may 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 a brown discoloration of the teeth. Infants of mothers treated with oxytetracycline during pregnancy may develop discoloration of the teeth. Some other chronic effects of oxytetracycline include increased sensitivity to the sun, wheezing and asthmatic attack. Toxicological studies indicate that this drug is not mutagenic, carcinogenic and terratogenic. Sixty percent of ingested dose absorbed from Gastro-intestinal tract and widely distributed in the body, particularly to liver, kidney, bones and teeth. Little metabolism of this drug in

humans or animals and it was primarily excreted in the urine. Oxytetracycline did induce antibiotic resistance in coliforms in the human intestine (JECFA, 1990).

Beta-lactam antibiotics usually cleared rapidly from the blood via the kidneys and excreted into the urine (residues in kidney and liver are about 100 times higher than muscles). Allergic reactions are the determining factor for safety evaluation of residues. Allergy to penicillin varies from 3–10% in different populations but still there is no evidence that penicillin residues in food caused sensitization. However, some cases of persons with known sensitivity suffering allergic reaction are recorded. Estimate shows that 10 IU (0.6 µg) of penicillin could cause an allergic reaction in a sensitive individual; 0.01 IU/ml of milk in a very sensitive individual (Waltner-Toews and McEwen, 1994). Two cases of anaphylactic reactions with known hypersensitivity to penicillin, steak (in 1984) and pork (in 1972) were recorded (Woodward, 2004).

Enrofloxacin a fluoroquinolone antibiotic is acts by inhibition of bacterial DNA gyrase. Embryo lethality and terratogenicity of fluoroquinolone antibacterials in rats and rabbits has been suggested (Guzman *et al*., 2003). Chromosomal aberrations evaluated in cultures of human peripheral lymphocytes from eight healthy donors, exposed to the antimicrobial enrofloxacin or to its major metabolite ciprofloxacin suggested a genotoxic effect of enrofloxacin and ciprofloxacin (Gorla *et al*., 1999). It is also associated with increased photosensitivity. The Food and Drug Administration's Center for Veterinary Medicine has proposed to withdraw approval for use of the fluoroquinolone antimicrobial, enrofloxacin, in poultry. The decision is not based on drugs direct toxicity but on potential for increasing human pathogen resistance.

Lemus (2008) suggested that antibiotic residues that may be present in carcasses of medicated livestock could pass to and greatly reduce scavenger wildlife populations. They surveyed residues of the quinolones enrofloxacin and its metabolite ciprofloxacin and other antibiotics (amoxicillin and oxytetracycline) in nestling vultures in central Spain. They found high concentrations of antibiotics in the plasma of many nestling cinereous (57%)

and Egyptian (40%) vultures. Enrofloxacin and ciprofloxacin were also found in liver samples of all dead cinereous vultures.

This is the first report of antibiotic residues in wildlife. They provide evidence of a direct association between antibiotic residues, primarily quinolones, and severe disease due to bacterial and fungal pathogens. Results indicate that, by damaging the liver and kidney and through the acquisition and proliferation of pathogens associated with the depletion of lymphoid organs, continuous exposure to antibiotics could increase mortality rates, at least in cinereous vultures. If antibiotics ingested with livestock carrion are clearly implicated in the decline of the vultures in central Spain then it should be considered a primary concern for conservation of their populations.

2.22 Antibiotic Resistance

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. If even at a large dose, the antibiotic is not effective in treating an infection, then the microorganism that is responsible for the infection is declared as resistant to that antibiotic. Antibiotic resistance is a global public health concern today. The U.S. Centers for Disease Control and Prevention has described antibiotic resistance as “one of the world’s most pressing health problems”, because “the number of bacteria resistant to antibiotics has increased in the last decade and many bacterial infections are becoming resistant to the most commonly prescribed antibiotic treatments.” The World Health Organization (WHO) has identified antibiotic resistance as “one of the three greatest threats to human health.”

Several WHO consultations and other expert bodies have identified links between antibiotic use in animals and the emergence of mainly food-borne bacteria which are resistant to important antibiotics which are used in treating infectious diseases in humans. In December 2003, an expert workshop was jointly convened by the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE) and the World Health Organization (WHO) to make a scientific assessment of resistance risks arising from non-human use of antibiotics/antimicrobials. The

workshop concluded that “there is clear evidence of adverse human health consequences due to resistant organisms resulting from residues of antimicrobial agents in food of animal origin in excess of the agreed acceptable maximum residue levels (MRLs).

Drug use in livestock is implicated in antimicrobial resistance in humans because many antimicrobial drugs used for livestock are the same as or similar to drugs used for humans. Some pathogens can pass from livestock to humans, either directly through contact (Feinman, 1979; Fey *et al.,* 2000; Holmberg *et al*., 1984) or through food products that are improperly processed, handled, or prepared. Some food borne illnesses in humans caused by resistant pathogens have been traced to livestock products (Gashe and Mpuchane, 2000; USDA, 1997; White *et al.,* 2001) and have been linked to live animals on farms.

In recent years, more evidence has emerged of an association between use of antibiotic agents in food animals and antibiotic resistance among bacteria isolated from humans. The medical consequences of resistance acquisition in bacteria of animal origin are highlighted by the following examples

Salmonella

There is direct evidence that antimicrobial used in animals are responsible for development of antimicrobial-resistant mainly in nontyphoid Salmonella serotypes. These bacteria have been transmitted to humans in food or through direct contact with animals. Antimicrobial resistance limits the therapeutic options available to veterinarians and physicians for the subset of clinical cases of nontyphoid Salmonella which require treatment. A recent example is a clone of *S. typhimurium* DT104, resistant to tetracyclines ampicillin, streptomycin, chloramphenicol and sulphonamides, which have become prevalent in many countries. An outbreak of human nalidixic acid-resistant *Salmonella typhimurium* DT104 infection in Denmark was traced to a pig farm. Another outbreak of the same infection, reported in the United Kingdom, was traced to a dairy farm where fluoroquinolones had been used on the cattle a month before the outbreak. Following the introduction of

fluoroquinolones for use in food-producing animals, the emergence of Salmonella serotypes with reduced susceptibility to fluoroquinolones in humans has become a cause for particular concern (Molbak *et al*., 1999).

Campylobacter

Following the introduction of fluoroquinolones for use in poultry there has been a dramatic rise in the prevalence of fluoroquinolone-resistant *Campylobacter jejuni* isolated in live poultry, poultry meat and from infected humans. Moreover, prior to any use in poultry, no resistant strains were reported in individuals with no previous exposure to quinolones. Fluoroquinolone-resistant *C. jejuni* has been associated with therapeutic failures in humans. In the United States, there was a marked increase in the proportion of domestically acquired Campylobacter infections that were fluoroquinolone resistant, following the first approved use of fluoroquinolones in food animals in 1995 (WHO, 2009).

Enterococci

The use of avoparcin as a growth-promoting feed additive in animal husbandry has contributed to the reservoir of transferable resistance genes to glycopeptides, including vancomycin, in the commensal enterococci of animals. Glycopeptide-resistant enterococci from animals can reach humans via the food chain. Although glycopeptide resistance genes have been shown to be widely disseminated, the extent to which the gene pool in animals contributes to the prevalence of glycopeptide-resistant commensal enterococci in humans has not been quantified. Glycopeptide-resistant enterococci cause serious infections in hospitalized immune-impaired patients. In this setting they contribute to increased morbidity and mortality, in part because of limited therapeutic options. This medical impact would be greatest in countries where vancomycin is used intensively.

There is concern that there will be increased dissemination of glycopeptide resistance genes to *Enterococcus faecalis* and their spread to other gram-positive organisms, particularly to multiresistant *Staphylococcus aureus* for which vancomycin is the drug of last resort. Due to the limited number of agents available for the treatment of glycopeptide-resistant

enterococci, antimicrobial agents not previously used in humans are being sought, including drugs from classes currently used as growth promoters in animals. Therefore the selection of further resistance in enterococci is undesirable, e.g., streptogramin resistance due to use of virginiamycin as a feed additive in animals.

Escherichia coli

Multiresistant *Escherichia coli* have been evolved by the use of broad spectrum antimicrobials in both livestock and humans. The development of antimicrobial resistance in *E. coli* creates problems due to their high propensity to disseminate antimicrobial resistance genes. Resistance genes have been traced from *E. coli* in animals to *E. coli* in humans. Certain *E. coli* are food borne pathogens and most of these strains are currently susceptible to antimicrobials.

As a consequence of decades of widespread use in the United States, resistance to the AGPs is very common in pathogenic and commensal bacteria from food animals. For example, the prevalence of resistance to tetracyclines, sulfonamides and beta-lactams among fecal *E. coli* from pigs and poultry is typically greater than 20%, and in some cases greater than 90% (Alali *et al*., 2008; Smith *et al.,* 2007). Importantly, AGPs also exert selective pressure to other antimicrobials of great importance to human medicine through the process of co-selection (Aarestrup *et al*., 2008; WHO, 1997). These resistant bacteria may colonize or cause infections in people exposed through contaminated food, by direct contact with infected animals, or indirectly through contaminated water or other environmental sources. Importantly, some of these bacteria that acquire resistance determinants in animals (e.g. *Enterococcus faecium, E. coli*) may colonize humans and share these genes with other human pathogens. In some cases, these altered pathogens may spread to other people in hospitals or other settings, in the face of additional antibiotic selection pressures in people (Barza, 2002).

The WHO, in fact, has recommended that antibiotics which are also licensed in human medicine should not be used any more as growth promoters in livestock (WHO, 2003). An EU resolution to this effect was put in

place in 1999. Since then, studies from Denmark, Germany and Italy have shown a significant reduction in Vancomycin-resistant Enterococci isolations from poultry and poultry-derived food products. Some European member states (such as Denmark) have, with insignificant or no consequence either on disease rates in animals or on meat market prices, voluntarily suspended the use of all growth promoters irrespective of their human health importance.

2.3 Detection of antibiotics residue by microbial inhibition assays

The detection of antibiotic drug residues in edible animal products has traditionally been performed by microbiological techniques. Microbial growth inhibition is the basis of screening methods for monitoring the presence of antibiotic residues in foods of animal origin (Hussein, 2004). They are based on the specific reaction between a susceptible organism (generally bacteria) and the antibiotic present in the sample. These methods are qualitative or semi-quantitative methods and are used as the mainstream screening methods for systematic detection of antibiotic residues in food. They allow detection of a wide spectrum of antibiotics in the sample and identify the specific antibiotic groups (Aerts *et al*., 1995; Haasnoot *et al*., 1999). The aim of the control of residues is to prevent residues in food exerting an undesirable effect on humans. Hence,

Some advantages of these assays are their reliability, cost-effectivity and simplicity. Moreover, a high number of them are commercially available (Kozarova *et al*. 2009). However, microbial inhibition cannot identify antibiotics and their precision appears to be variable. Furthermore, these tests often lack the specificity and sensitivity required for residue detection at maximum residue levels (MRLs), may be affected by non-specific inhibitors and often have a 24 h or more incubation time (Wibawa *et al*., 2002).

Concurrent but independent work by many investigators led to development of a plethora of methods based on inhibition of the bacterial growth by the antibiotics present in livestock products. Microorganisms such as *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* (Wolin and Kosikowski, 1958), *Bacillus mesentericus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Candida tropicalis*, *Lactobacillus bulgaricus*, *Micrococcus*

flavus, *Pseudomonas syringae*, *Micrococcus luteus*, *Staphylococcus epidermidis*, *Streptococcus* (Whitehead and Cox, 1956) have all been employed with varying success for determining the presence of various antibiotics residues in food of animal origin (Botsoglou and Fletouris, 2000).

Ellerbroek (1991) compared the sensitivity of *B. Subtilis* and *E. coli* towards enrofloxacin, ciprofloxacin, and flumequine. He proposed an extension of the German three-plate (*B. Subtilis*) method with *E. coli*, which was found to be 3–30 times more sensitive depending on the quinolone residue. Similarly, Choi *et al*. (1999) compared several other *E. coli* strains with *B. Subtilis* ATCC 3491, which was the official test organism for antibiotic screening in Canada. Besides enrofloxacin, ciprofloxacin, and flumequine, the study included also sarafloxacin and difloxacin. For all these residues *E. coli* ATCC 128 appeared to be superiorly sensitive and this organism was recommended for supplementing the existing microbial screening tests.

Petrovic et al. (2006) used Microbiological method – plate pH 8.0 with Escherichia coli NCIMB 11595 gave positive results in all the samples which had residues above MRL values. Another comparative study evaluated the susceptibility of the same organisms as in (Ellerbroek, 1991) for ten different quinolones (Okerman *et al*., 2007). Only difloxacin appeared to be detected more sensitively using *B. subtilis* as the test organism. The paper also shows the differences between growth medium at pH 6 and pH 8. Detection of naldixic acid, flumequine, oxolinic acid, and difloxacin appeared to be optimal at pH 6; for the others pH 8 is favorable. It was concluded that the addition of an *E. coli* pH 8 test is the best option to include in existing screening methods.

Alla *et al.,* (2011) detect the presence of antibiotics residues in Ghanawa slaughterhouse, Khartoum State using one plate test (O. P. T.). The test organism was *Bacillus subtilis* (strain ATCC6633). The sample was considered to be positive when the inhibition zone was 2 mm and more, doubtful when it was 1 to 2 mm and negative when it was less than 1 mm. Out of 300 animals 52 (17.33%) showed positive results in one or more of their organs. Out of 300 kidneys tested, 30 (10%) showed positive result, while out

of 300 livers tested, 23 (7.66%) were positive, and out of 300 muscles tested 9 (3%) were positive.

Ibrahim *et al.* (2010) screened 50 slaughtered animals at Sokoto metropolitan abattoir for antibiotic residues in meat by using microbial inhibition test using *Bacillus stearothermophilus* and *Staphylococcus aureus* and reported that 44% of the slaughtered animals were positive. Penicillin (14%) was the drug with the highest rate of occurrence followed by tetracycline (8%) and streptomycin (4%) in samples positive on a single plate. Multiple antibiotic residues were found from 9 (18%) slaughtered animals.

Karraouan *et al*. (2009) describes a microbiological method for the detection of antibacterial substances in poultry muscle. The method is based on the growth inhibition of *Bacillus subtilis* on an agar medium at a pH of 6.0, 7.2, and 8.0, *Bacillus cereus* on an agar medium at a pH of 6.0, M. luteus on agar medium at a pH of 6.0 and 8.0, and *Escherichia coli* on agar medium at a pH of 7.2, and on the use of confirmatory solutions (Pase, Paba, MgSO4) for the identification. The proposed method detects the main antibiotic groups: β- lactams, tetracyclines, aminoglycosides, macrolides, and quinolones at low levels. The results are obtained in 18-24 h. This technique can be used as a screening method for the detection of antibiotics in animal tissue, but a more specific method would be necessary for full identification of antimicrobials in screening positive samples.

Musser *et al.* (2001) investigated the potential for antibiotic residues in calves from consuming milk containing penicillin G or amoxicillin. Six calves were fed milk replacer, 6% body weight twice daily, containing 0.293, 2.92, or 5.85 µg of penicillin/ml (ppm) G or 0.25, 1.0, or 2.0 pg of amoxicillin/mi for three consecutive feedings. Urine and blood samples were collected after each feeding. Serum and urine samples were tested with a microbial receptor assay and a microbial growth inhibition assay to indicate potential drug residues. Penicillin G and amoxicillin were detected in the serum and urine of several calves 3 h after drinking spiked milk replacer. Possible violative drug residues in the calves were detected by the microbial growth inhibition assay up to 15 h after drinking spiked milk replacer. Penicillin

G, but not amoxicillin, could be detected in urine 24 h after the final feeding of spiked milk replacer.

Smither *et al*., (1980) conducted Investigations between 1977 and 1979 to assess the performance of microbiological tests for detecting and identifying residues of therapeutic-type antibacterial substances in meat and offal. Of the 5442 home-produced meat samples examined, 34 (0.63 %) showed inhibitory activity in the screening test, which used *Bacillus subtilis* and *Micrococcus luteus* as indicator organisms. Identification by electrophoretic and thin-layer chromatography/bio-autography techniques confirmed that only two of the 34 screen failures were due to true antibacterial residues.

2.4 Determination of antibiotics residue by chromatographic methods

The identity and quantity of the residue in a suspected sample cannot be determined with a screening test. Hence the decision about the compliance of a sample cannot be based on 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 mixtures 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 the past six decades, chromatography has been extensively applied to all branches of science. 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 tremendously impacted chemistry-related sciences. More impressively between 1937 and 1972, a total of 12 Nobel Prizes were based on work in which chromatography was a key tool.

In all chromatographic separations, the sample is carried by the mobile phase, which may be a gas, a liquid, or a supercritical fluid. The mobile phase is then percolated through an immiscible stationary phase that is fixed

on a solid substrate. When the sample passes through the stationary phase, species are retained to varying degrees as a result of the physicochemical interaction between the sample species and the stationary phase. The separation of species appears in the form of bands or zones resulting from various retentions. Chemical information can thus be analyzed qualitatively and/or quantitatively on the basis of these separated zones.

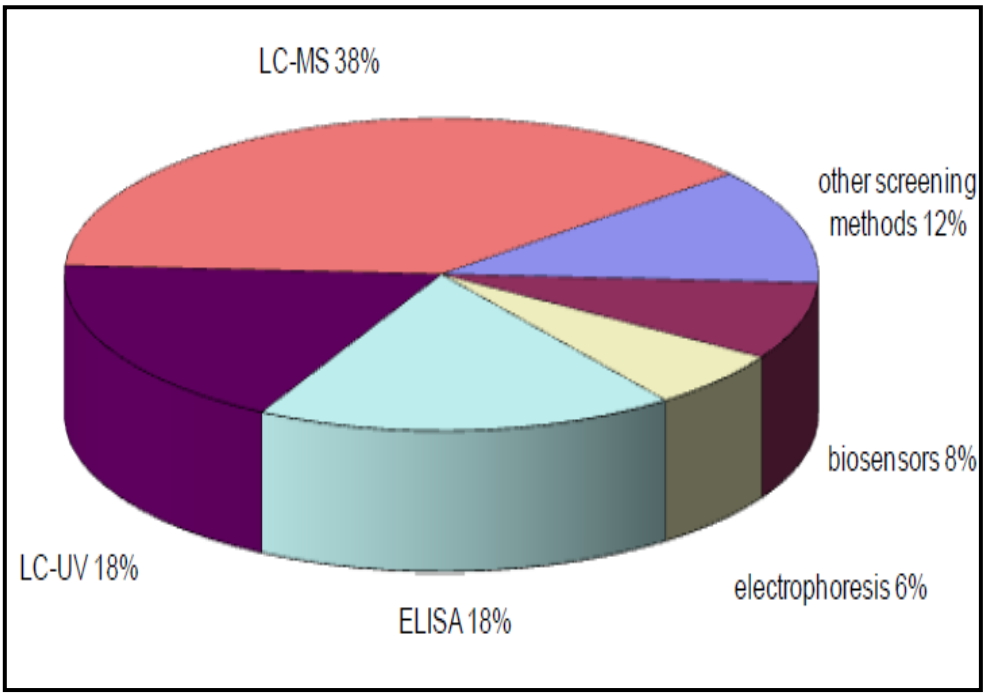

Fig. 3: Distribution of the analytical methods used for the determination of antibiotics in food (Chafer-Pericas *et al.,* 2010)

High Performance Thin layer chromatography (HPTLC) is a sensitive and reliable method for monitoring low amounts of different biological and chemicals. HPTLC allows the qualitative and quantitative detection of multi-residues in meat. The plates are sprayed with an appropriate chromogenic reagent or viewed under UV light for visualisation of compounds. Detection by fluorescence is also applied. Quantification is achieved by measuring the relative intensity of the spot verses that of the internal standard by scanning densitometry. Modern HPTLC has been automatised at a high level. Determination of drug residues in food is an important application of HPTLC. Milk and meat samples (beef, pork, fish, poultry, etc) can be subjected to analysis of multiclass, multiresidue screening.

Shareef *et al*. (2009) studied the antibiotic residues in stored poultry products particularly in liver, breast and thigh muscle samples using Thin Layer Chromatography. A total of 75 samples stored poultry products; liver, breast and thigh muscle samples, were tested for the presence of four antibiotics residue; oxytetracycline, sulfadiazine, neomycin, and gentamycin using TLC. The results revealed 39 (52%) positive samples. From 25 samples of each of liver, breast and thigh muscle samples tested, 7 (28%) of liver and breast muscle were positive for sulfadiazine and oxytetracycline while 7 (28%) of thigh muscle were positive for oxytetracycline and 4 (16%) samples were positive for sulfadiazine. No neomycin or gentamycin residues were detected on TLC plates in all samples tested. Oxytetracycline was the most predominant antibiotic detected (28%), among the four studied antibiotics and followed by Sulfadiazine (24%). Liver and breast muscle had the highest percentage of antibiotic detected (56%), followed by for thigh muscle (44%).

Shahid *et al.,* (2007) conducted a study to monitor the status of oxytetracycline (OTC) residue in poultry meat in Rawalpindi and Islamabad area of Pakistan. The preliminary screeninfg of samples for the presence of antibiotic residues was performed by a microbiological assay using *Bacillus subtilis* as 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 were greater 91% with RSD values between 1.64 to 2.07% while the limit of detection (LOD) 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 then the recommended maximum residue level (0.2, 0.6 and 1.2 µg/gm) for muscles, liver and kidney, respectively.

Biswas *et al.* (2007) developed a simple, rapid and sensitive method for residue monitoring of oxytetracycline, tetracycline and chlortetracycline in buffalo meat samples. The principal steps involved extraction in McIlvaine buffer (pH 3.85) followed by a solid phase clean up step. In HPLC, a reversed phase C8 (RP-C8) column was used and compounds were separated at 35°C using a mobile phase of 0.01 M oxalic

acid buffer (pH 1.6)/acetonitrile/methanol (77:18:5, v/v/v) at a flow rate of 0.6 mL/min. A wavelength of PDA detector was set at 355 nm. The detection limit of the method was calculated to be 0.031 µg/g and the minimum detectable quantity was found to be 0.062 µg/g.

Naeem *et al*. (2006) conducted a study for the estimation of quinolones in 150 poultry samples (120 samples of liver, kidney and muscles and 30 samples of egg) purchased from local markets in Lahore, Pakistan. The quinolones included in the study were ciprofloxacin, enrofloxacin, levofloxacin, norfloxacin, ofloxacin, flumequine, oxolinic acid and nalidixic acid. The poultry products included muscle, liver, kidney and egg. Ten gram of each of the samples of liver, muscle, egg and five gram of kidney samples were used for extraction and High Performance Liquid Chromatography (HPLC) system is used for determination of quinolones. The result indicated that 58 to 85% of ciprofloxacin and 55 to 92% samples of enrofloxacin violated the regulation. They concluded that enrofloxacin occur most abundantly and widely in the products and liver and kidney are the most contaminated part of chicken than muscle and egg. They found enrofloxacin concentration in the range of 3.10 to 364 µg kg^{-1} in liver.

Tajick and Shohreh (2006) worked on detection of antibiotic residue in chicken meat using TLC. Nowadays antibiotics are applying for control of infectious diseases. Incorrect use of these drugs deposits some residue in product. This research highlights the importance and existence of antibiotics residue in meat. In this survey 10 grams of chicken meat crashed and squeezed in 10 ml ethanol. After clarifying by centrifuge the solvent evaporated totally. After loading and running on silica F256 plates, the chromatograms observed on UV light. The results showed more than 50% of meat samples had noticeable antibiotics residue.

Amjad *et al.* (2005) done analysis and comparison of selected residual antibiotics in broiler chicken available in local market. The broiler samples included muscle, kidney and liver. The quinolones included in this study were, oxolinic acid, nalidixic acid, flumequine, enrofloxacin, norfloxacin and ciprofloxacin. The intertissue/organ comparison within each analytical

technique and intermethod comparison of results obtained by HPLC, UV spectroscopy and ion association complex techniques were made. TLC was used to separate and identify the quinolone residues. HPLC with ODS column and UV detector and UV/ visible spectroscopy were used for quantification of the residues. Good compatibility of the spectrophotometric results was found with those of high pressure liquid chromatography.

Ramirez *et al*. (2003) developed an analytical method to identify and quantify multiple antibiotic residues in cow's milk by combined application of high-performance thin-layer chromatography (HPTLC) with bioautography. The test microorganism used for bioautography was *Bacillus subtilis* ATCC 6633. Antibiotic residues were extracted with acetonitrile, fat eliminated with petroleum ether and residues isolated with dichloromethane The sensitivity of the method guarantees the detection of the above-mentioned antibiotics at levels below maximum residue limits (MRL) allowed for milk. Percentage recoveries ranged between 90 and 100%, with coefficients of variation between 7.2 and 21.3%. Major improvements of this methodology over TLC/ bioautography were: better sensitivities for antibiotics, easier to handle materials, smaller amounts of bacteriological media and developing solvents, and reduced spotting amounts of standard and milk extracts.

Oka *et al*. (2000) designed an improved method for HPTLC analysis of tetracyclines. The plate is predeveloped with a saturated Na_2EDTA aqueous solution and is activated before applying the sample. Using this pre-developing technique, they have reported the successful separation of eight TCs on a silica gel high-performance TLC plate with a solvent system of chloroform–methanol–5% Na_2EDTA (65:20:5, lower phase) and applied this TLC technique to the analysis of the eight residual TCs.

Choma (2000) determined four tetracyclines in milk by a simple thin layer chromatographic method. They perform HPTLC of tetracyclines on silica gel (with concentration zone, impregnated with 5% aqueous Na_2EDTA solution) with the lower layer of chloroform: methanol: 5% aqueous Na_2EDTA 13:4:1 after a predevelopment with n-hexane and acetone to remove lipid

fractions. Detection of fluorescent spots was done at 254 and 366 nm. Detection limit is found to be 0.1 µg (two fold development with mobile phase).

Luo and Ang (2000) determined trace levels of amoxicillin residues in animal tissues by liquid chromatography (LC) with fluorescence detection. An improvedsolid-phase extraction (SPE) procedure requiring less flammable solvent (diethyl ether) was developed for sample preparation. Muscle samples of beef, pork and chicken were extracted with a phosphate buffer followed by the modified SPE procedure for cleanup and concentration prior to the LC–fluorescence analysis. Average recoveries of fortified amoxicillin at 5, 10, and 20 mg/kg ranged from 83.9 to 85.8% in beef, 86.1 to 88.1% in pork and 81.7 to 82.9% in chicken.

Argekar *et al*. (1996) develop method for HPTLC of six fluoroquinolones (ciprofloxacin, enrofloxacin, lomefloxycin, norfloxacin, ofloxacin and perfloxacin) on silica with butanol-ethanol-ammonia 20:5:11. Densitometry at 280 nm for ciprofloxacin, enrofloxacin, norfloxacin, perfloxacin, and 285 nm for lomefloxycin. The linearity range was found to be 10-150 ng. R_f values were in the range of 0.35 to 0.40 and this HPTLC method was found to be comparable with official methods

Oka *et al*. (1987) develop method of TLC of residual oxytetracycline, tetracycline, chlortetracycline, doxycycline, methacycline, dimethylchlortetracycline and monocycline in honey on silica and RP-8 silica with chloroform- methanol-5% aqueous Na_2EDTA 65:20:5 (lower phase) and methanol – acetonitrile – 0.5M aqueous oxalic acid 1:1:4 (pH 3.0), respectively. Observation done under UV at 360 nm. Detection limit of this method was found to be 0.1 ppm. .

Neidert *et al.* (1987) develop an analytical method for the identification of the residues from 14 commonly used antibiotics. The technique is based on selective tissue extraction followed by thin layer chromatography/bioautography. Antibiotic residues are extracted from the tissues with methanol and methanol-HCl (98 + 2). The methanol extract is further extracted with chloroform to isolate groups of antibiotics. The extracts are spotted onto TLC plates and developed in suitable solvent systems.

Developed plates are placed on set medium seeded with *Bacillus subtilis* and a bioautograph is produced. The locations of zones of inhibition are used to identify antibiotic residues. Recoveries of antibiotics were quantitative, while the effect of naturally inhibiting components of the matrix was minimized. The sensitivity of the method can be adjusted through minor modifications, which allows its use in routine regulatory analysis.

2.5 MAXIMUM RESIDUE LIMITS

A number of national and international organizations are involved in the legislation on residues of veterinary drugs in foods. Countries lend to follow their own guidelines. The Food and Agricultural Organization (FAO) and Word 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 (Oil-) 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 Young, 1993). MRL is defined as maximum concentration of residue 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 a 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 Codex maximum residue limits (MRLs) set for OTC/TC/CTC (alone or combination) are 0.1 µg/g in muscle tissues.

Table. 1: Overview of European Union maximum residue limits (µg/ kg)

Pharmacologically active substance	Animal species	Commission regulation	Target tissues		
			Muscle	Liver	Kidney
Amoxicillin	All food producing species	508/1999	50	50	50
Oxytetracycline	All food-producing species	508/1999	100	300	600
Enrofloxacin	Porcine, poultry, rabbits	1181/2002	100	200	300

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 organizations in the legislation of veterinary drugs has made it very difficult to standardize control practices and harmonize tolerance levels internationally in a 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 Young, 1993). Therefore, it has been proposed that the ADI is better choice for determination of food safety rather than MRL (Brynes et *al.,* 1996).

Nisha (2008) reported deposition of antibiotic residues in meat, milk and eggs and concluded that if use of antibiotics is necessary as in prevention and treatment of animal diseases, a withholding period must be observed until the residues are negligible or no longer detected.

2.6 EFFECT OF COOKING PROCESSES

For the past 50 years, many researchers have been interested in evaluating whether antibiotic residues can be destroyed by cooking procedures, pasteurization, or canning processes (Ibrahim and Moats, 1994; Rose *et al*., 1995; Hassani *et al*., 2008). Residues of a range of antibiotics have varying degrees of stability during cooking and, therefore, the cooking influences the level of risk posed by such residues (Rose *et al*., 1999). Since the most of food producing animals are always cooked before consumption and the variations in antibiotics levels in the tissue are dependent on the type of cooking (Lolo *et al*., 2006), more findings about the effect of cooking on antibiotic residues are needed to accurately determine consumer exposure to these drugs.

Javadi *et al.* (2011) determine the effects of different cooking processes like boiling, roasting and microwaving on Enrofloxacin residues in muscle, liver and gizzard tissues of broiler chickens. Each of the chicks was fed by water and food with 0.05% of Enrofloxacin in their drinking water for 5 consecutive days. Then, three locations were sampled aseptically from each carcass: breast muscle; liver and gizzard. Enrofloxacin residue was analyzed using microbial inhibition method by plates seeded with *Escherichia coli*. After doing different phases of the test on raw samples, the positive raw samples were cooked by various cooking procedures and the cooked samples were surveyed with similar method again for the presence of residue. The results showed the reduction in concentration of enrofloxacin residue after different cooking processes. The most reduced residue in cooked meat and gizzard samples related to boiling process and the cooked liver samples was the roasting process. The highest detectable amount of residue belonged to microwaving process in all cooked samples. Regarding to the results of this study, it was concluded that, cooking processes cannot annihilate total amounts of this drug and it can only decrease.

Roca *et al.* (2010) determine the effect of different temperatures and heating times on the concentration of quinolones by employing liquid chromatographic equipment analysis with fluorescence detection. In order to determine the thermo-stability of these compounds, the first-order kinetic model was applied, and the activation energies, half-lives, and percentages of degradation of each compound were calculated. Results showed that quinolones are very resistant to different heat treatments with maximum losses of concentration of 12.71% for ciprofloxacin and 12.01% for norfloxacin at 120 °C and 20 min. The high stability of quinolones represents a significant risk to human health because the residues of these antibiotics can remain in milk after heat treatment and, therefore, can reach the consumers.

Zorraquino *et al.* (2008) analyze the effect of different heat treatments (40°C for 10 min, 60°C for 30 min, 83°C for 10 min, 120°C for 20 min, and 140°C for 10 s) on milk samples fortified with three concentrations of nine ß-lactam antibiotics (penicillin G: 3, 6, and 12 µg/liter; ampicillin: 4, 8, and 16 µg/liter; amoxicillin: 4, 8, and 16 µg/liter; cloxacillin: 60, 120, and 240 µg/liter; cefoperazone: 55, 110, and 220 µg/liter; cefquinome: 100, 200, and 400 µg/liter; cefuroxime: 65, 130, and 260 µg/liter; cephalexin: 80, 160, and 220 µg/ liter; and cephalonium: 15, 30, and 60 µg/liter). The method used was a bioassay based on the inhibition of *Geobacillus stearothermophilus*. The results showed that heating milk samples at 40°C for 10 min hardly produced any heat inactivation at all, while the treatment at 83°C for 10 min caused a 20% loss in penicillin G, 27% in cephalexin, and 35% in cefuroxime.

The thermostability parameters of three tetracycline antibiotics at high and ultrahigh temperatures (110-140^{o}C) have been investigated by Hassani *et al.* (2008). The thermal degradation of the three antibiotics followed a first-order reaction kinetic within the 1.5-2 log(10) cycles investigated. The temperature dependence of the DT values was similar for the three molecules (z=28+/-2 degrees C). DT values of doxycycline were approximately 1.5 and 3 times higher than those of tetracycline and oxytetracycline, respectively. Low-temperature-long-time treatments (conventional sterilization) destroy >98% of the initial concentration of the residues of the three antibiotics, high-temperature-short-time treatments (UHT) would leave unaltered residues in the 50-90% range.

Lolo *et al*. (2006) conducted a study to determine the effect of different cooking processes (microwaving, roasting, boiling, grilling and frying) on naturally incurred enrofloxacin residues in chicken muscle. Enrofloxacin and its metabolite, ciprofloxacin, were analysed using a validated LC–MS method with limits of detection (LOD) and quantification (LOQ), respectively, of 2 and 5 ng/g quinolones in muscle samples. The RSD with naturally incurred roasted chicken breast was 9.18% at a concentration of 11±1.01ng/g (*n*=6). In water, enrofloxacin remained stable for 3 h when heated at 100°C. It was concluded that residue data from raw tissue are valid for estimation of consumer exposure to this drug, as well as the ADI calculations because cooking procedures did not affect enrofloxacin residues, which remained stable during heating. However, there was an apparent decrease in quinolone concentration in tissue because some was lost by exudation into the liquid used for cooking. Conversely, for a cooking procedure with water loss, there was an apparent increase in residue concentration.

Al-Ghamdi *et al.* (2000) collected 247 chicken-muscles, 719 chicken-liver and 630 eggs samples over 49 inspection visits to 33 broiler and 5 layer farms in the eastern province of Saudi Arabia. OTC was detectable in 78.3% of the farms and 62.4% of raw chicken-muscle samples. Mean concentrations of OTC in raw muscle was found to be 1.45±1.14 µg/g which exceeded the muscle MRL value (0.10 µg/g) in all 18 OTC positive farms. After cooking, the overall mean concentration of this drug in muscle reduces to 1.02±0.70 and was detected in 73.9% farms and 58.1% samples only.

Rose *et al.* (1996) studied the heat stability of oxytetracycline (OTC). Results showed that the drug was unstable in water at 100 °C with a half- life of about 2 min, but more stable in oil at 180° C where the half- life was about 8 min. The effect of a range of cooking processes including microwaving, boiling, roasting, grilling, braising and frying on OTC residues in incurred animal tissues was investigated. Substantial net reductions in OTC of 35–94 % were observed, with temperature during cooking having the largest impact on the loss. Migration from the tissue into the surrounding liquid or meat juices was observed during the cooking processes. The findings of this investigation showed that the effect of cooking on residues of OTC should be

considered before data obtained from measurements on raw tissue are used for consumer exposure estimates and dietary intake calculations.

Ibrahim and Moats, (1994) studied the effect of cooking procedures on oxytetracycline residues in lamb muscle. Oxytetracycline was administered intravenously in the lambs 4 hr prior to slaughter. Muscle samples were collected and mixed to ensure uniformity. Residues were determined by HPLC analysis. When the meat was cooked in boiling water, oxytetracycline was reduced by 95% in 30 min. They correlate the degradation in the residue quantity with the final temperature reached during cooking procedure.

O'brien *et al.* (1981) determine the effect of cooking and cold storage on biologically active antibiotic residues in meat. The results indicate that there was on average less than 10 % reduction in the annular diameter size, the range being from no reduction (chloramphenicol) to 15% (oxytetracycline).

Yonova (1971) in his study showed that the inactivation of oxytetracycline in poultry meat and eggs depends on the amount present and duration of cooking and for the full inactivation of residual oxytetracycline in poultry meat it is necessary to cook for 60 min or more.

3. MATERIAL AND METHODS

Location and technical programme of work

The work was conducted in Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Madhya Pradesh Pashu Chikitsa Vigyan Vishwa Vidyalaya, Jabalpur, Madhya Pradesh, India.

Surveillance study

No authentic data has been made available from the government and other non government agencies on therapeutic and growth promoting uses of antibiotics in poultry. The antibiotics which are more frequently used, or at least which are detected most often in the carcasses or meat, information was gathered by conducting a surveillance study pertaining to use of antibiotic in various poultry farms located in and around Jabalpur district. The information was also gathered to shortlist most commonly and frequently used three antibiotics in chickens for further investigations and hence oxytetracycline, enrofloxacin and amoxicillin were short listed for determination of their residual concentration in samples of muscle, liver and kidney of chickens.

Collection of meat samples

For this study, a total of 180 poultry meat samples including muscle, liver and kidney 60 each were collected randomly from slaughtered birds of selected target areas (government and private sector slaughter houses/poultry farms) located in and around Jabalpur. Approximately 10 g of muscle, liver and kidney sample, each of the same bird were aseptically collected for detection of antibiotic residues.

Table 2: Design of experiment for detection of antibiotics residues

Group	Target areas	Tissue Samples	Number of samples	Total Number of samples
T_1	Sale Counter Veterinary College, Jabalpur	Muscle	12	36
		Liver	12	
		Kidney	12	
T_2	Government Slaughter House, Jabalpur	Muscle	12	36
		Liver	12	
		Kidney	12	
T_3	Military Slaughter House, Jabalpur	Muscle	12	36
		Liver	12	
		Kidney	12	
T_4	Joy poultry farm, Jabalpur	Muscle	12	36
		Liver	12	
		Kidney	12	
T_5	Ansari Poultry Farm, Jabalpur	Muscle	12	36
		Liver	12	
		Kidney	12	
Total number of samples				180

Transport and storage of meat sample

Meat samples were collected in clean sample collection container. After notation of samples characteristics they were transported to the laboratory in thermo-cooled container jacket with ice and were stored in refrigerator at 0^0C till processing.

The chemicals and techniques used for extraction, detection and quantification of residual concentration of oxytetracycline, enrofloxacin and amoxicillin have been mentioned below:

Chemicals

The following chemicals were employed during the investigation:

(i) Methanol: HPLC grade, Chromasolv® (Sigma - Aldrich)

(ii) Chloroform : HPLC grade, Chromasolv® (Sigma - Aldrich)

(iii) Butanol : HPLC grade, Chromasolv® (Sigma - Aldrich)

(iv) Dimethyl sulfoxide : HPLC grade, Chromasolv® (Sigma - Aldrich)

(v) Acetic acid: HPLC grade, Chromasolv® (Sigma - Aldrich)

(vi) Water : HPLC grade, Chromasolv® (Sigma - Aldrich)

(vii) Disodium ethylene diamine tetra acetate

(viii) Ninhydrin (GR, EI Merck)

(ix) Muller hington agar (HiMedia)

Screening of samples by microbial growth inhibition assay

Test organism

The following bacterial culture were procured from Institute of Microbial Technology (IMTECH), Chandigarh, and maintained at 4^{o}C in nutrient agar slants.

Table 3: Bacterial cultures used in microbial growth inhibition assay

S. No.	Bacteria	MTCC Cat. No.
1	*Staphylococcus aureus*	1144
2	*Streptococcus pyogens*	442
3	*Escherichia coli*	723
4	*Bacillus cereus*	1272

Test media

Muller Hinton agar (Hi-Media) in the form of dehydrated powder was used. Thirty eight grams of Muller Hinton agar was dissolved in 1000 ml of distilled water in a sterile conical flask. The medium was autoclaved at 121°C for 15 min. The medium was allowed to cool till 50°C. Twenty five

milliliters of the sterile culture medium was distributed for each Petri dish (diameter 90 mm) and the solidified agar was kept in refrigerator at 4°C.

Preparation of bacterial broth:

The cultured bacteria were inoculated in Brain Heart infusion nutrient broth tubes and incubated at 37°C for 24 hours for the experiment.

Preparation of discs:

Ten gram of each sample was mixed in 10 ml ethanol (96%), crushed and squeezed finely in a Chinese mortar. The solution was transferred to 15 ml centrifuge tubes and centrifuged at 7000 rpm for 10 minutes. The clear supernatant was transferred to fresh glass test tubes. Sterile discs (Hi-media) were soaked in these extracts in a sterile petridish for twenty four hours and were dried in laminar air flow. After drying the discs were used immediately for disc impregnation in the inoculated plates as described by Kirubaharan *et al.* (1999) with slight modifications.

Test procedures

The above prepared bacterial inoculums of *S. aureus, S. pyogens, E. coli* and *B. cereus* were evenly spread on sterile Muller Hinton Agar plates (Hi-media) as per the method described by Bauer *et al*. (1966) and microbial growth inhibition effect was studied by the disc diffusion method in these plates. The dried discs inoculated with Meat sample were kept at definite distance on each plate and a blank disc was used for negative control. After this all the plates were incubated at 37°C for 24 hours (Javadi *et al.,* 2011).

Interpretation of the test plate results

The presence of antibiotics was shown by the formation of growth inhibition zones around the disc after incubation of plates. Inhibition zones were measured by antibiotic zone inhibition reader (HiMedia). A positive raw sample is indicated by a complete inhibition of growth in an annular zone not less than 2 mm wide around the disc. Less than 2 mm of inhibitory zone indicated negative result (Myllyniemi *et al.,* 2001).

QUANTIFICATION OF POSITIVE SAMPLES

Apparatus

The HPTLC system (Camag, Muttenz, Switzerland) consisted of Linomat V autosprayer connected to a nitrogen cylinder, twin trough chambers (10 X 10 cm and 20 X 10 cm), Hamilton syringe (100µl), derivatization chamber, plate heater and TLC Visualizer. Precoated silica gel 60 F_{254} TLC plates (10 X10 cm and 20 X 20 cm) 0.2 mm thick (E. Merck KGaA, Darmstadt, Germany) was used as a stationary phase. Densitometric analysis was carried out using a TLC Scanner 4 with winCATS software.

STANDARDS:

The following antibiotics were used as standards:

(i) Oxytetracycline hydrochloride salt: (Sigma-Aldrich)
(ii) Enrofloxacin salt (Fluka-analytical)
(iii) Amoxicillin sodium salt: (Sigma-Aldrich)

1. OXYTETRACYCLINE

Oxytetracycline was the second of the broad-spectrum tetracycline group of antibiotics to be discovered. Oxytetracycline works by interfering with the ability of bacteria to produce proteins that are essential to them. Without these proteins the bacteria cannot grow, multiply and increase in numbers. Oxytetracycline therefore stops the spread of the infection and the remaining bacteria are killed by the immune system or eventually die.

Oxytetracycline is used to treat a variety of infections and is also used as a growth promoter in animals. Oxytetracycline can also be used to correct breathing disorders in livestock. Oxytetracycline is administered in a powder or through an intramuscular injection. Livestock producers apply oxytetracycline to livestock feed to prevent diseases and infections in cattle and poultry. However, some strains of bacteria have developed resistance to this antibiotic, which has reduced its effectiveness for treating some types of infection.

Physico-chemical properties of oxytetracycline

Molecular formula : $C_{22}H_{24}N_2O_9$.HCL

Molecular mass : 460.434 gm/mol

Half life : 6-8 hr

Excretion : Renal

NOAEL : 18 mg/kg body weight/day.

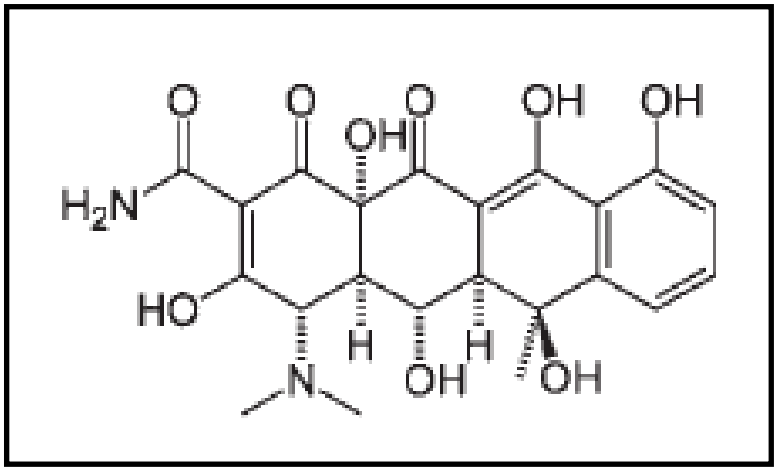

Fig. 4. Chemical structure of oxytetracycline

Antibiotic extraction:

1. Five gram of muscle/liver/kidney sample was blended in a high speed blender with 20, 20 and 10 ml of 0.1 M disodium-ethylene diaminotetraacetate (Na_2EDTA)-Mcllvaine buffer (33.62 gm of Na_2EDTA + 614.5 ml of 0.1 M citric acid + 385.5 ml of 0.2 M disodium hydrogen phosphate, pH 4.0); centrifuged each time at 4000 rpm for 5 min.
2. The supernatants were combined and recentrifuged at 4000 rpm. The filterate was passed through a C_{18}-cartridge pretreated with saturated aqueous Na_2EDTA.
3. The cartridge was washed with 20 ml of water and air dried by aspiration for 5 min. Oxytetracycline was eluted with 10 ml of ethyl acetate followed by 20 ml of methanol-ethyl acetate (5 : 95).
4. The elute was evaporated to dryness under reduced pressure at 30^{o}C and the residues were dissolved in 0.1 ml of methanol.

Preparation of silica plates:

The silica gel 60 F_{254} TLC plates (Merck, Germany) was pre-developed with saturated aqueous Na_2EDTA solution, air dried at room temperature for 1 hr and activated at 130^oC for 2 hr in hot air owen before use.

Standard preparation

Stock solution was prepared by weighing oxytetracycline (10 mg). Weighed powder was accurately transferred to a volumetric flask of 100 ml and dissolved in and diluted to the mark with methanol to obtain a standard stock solution of oxytetracycline (0.1 mg/ml) (Thangadu *et al.,* 2002).

Sample application

The standard (2-10µl) and samples were spotted on TLC plates in the form of narrow bands of length 6 mm with 8 mm distance from bottom and 15 mm from left margin. The samples were applied under continuous drying stream of nitrogen gas at constant application rate of 150 nl/s.

Mobile phase and migration

Silica plates were developed using mobile phase consisting of chloroform – methanol – 5% aq. Na_2EDTA (65 : 20 : 5). Linear ascending development was carried out in 10 X 10 cm twin trough chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 20 min. Ten milliliters of the mobile phase (5 ml in trough containing the plate and 5 ml in other trough) was used for each development and allowed to migrate a distance of 8 cm. After development, the TLC plates were dried completely.

Densitometric analysis and quantitation procedure

Densitometric scanning was performed on camag TLC scanner 4 in absorbance mode and operated by winCATS planar chromatography version 1.3.4. The source of radiation utilized was deuterium lamp. The spots were analyzed at a wavelength of 360 nm. The slit dimension used in the analysis was length and width of 5 mm and 0.30 mm respectively with a scanning rate of 20 mm/sec. These are selected as recommended by the

CAMAG TLC scanner 4 manual. It covers 70 – 90% of the application band length, which in the present case is 6 mm. The monochromator band width was set at 20 nm. Concentration of compound chromatographed were determined from the intensity of diffusely reflected light and evaluated as peak area against concentrations using linear regression equation.

2. ENROFLOXACIN

The quinolones are a group of synthetic antimicrobial agents that have a wide spectrum of action and high efficacy against various bacterial infections, especially Gram-negative bacteria but lesser against gram-positive cocci (Salehzadeh *et al.,* 2007). Also, they have been recommended for the treatment of urinary tract and enteric infections. The antibacterial activity of quinolones is based on the inhibitionof DNA-gyrase which leads to an unstable condensation of the DNA configuration of the bacterial DNA molecule during cell division (Xu *et al.,* 2006).

Enrofloxacin is a synthetic fluoroquinolone antimicrobial agent developed in the late 1980s exclusively for use in veterinary medicine. Enrofloxacin belongs to the early second or second generation of fluoroquinolone antimicrobials, which have bactericidal activity against *Enterobacteriaceae* and other Gram-negative bacteria and have some activity against certain Gram positive cocci (Martindale, 2005; Martinez *et al*., 2005). In veterinary medicine, it is administered orally to chickens and turkeys, for the treatment of infections of the respiratory and alimentary tract. The recommended doses are 10 mg enrofloxacin/kg bw/day for 3 to 10 days (chickens and turkeys) (Cupic *et al.*, 2002).

Physico-chemical properties of enrofloxacin

Chemical Name **:** 1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1, 4-dihydro- 4-oxo-3-quinolonecarboxylic acid

Molecular formula **:** $C_{19}H_{22}FN_3O_3$

Molecular Weight **:** 359.4 gm/mol

Half life **:** 4-6 hr

Excretion **:** Renal

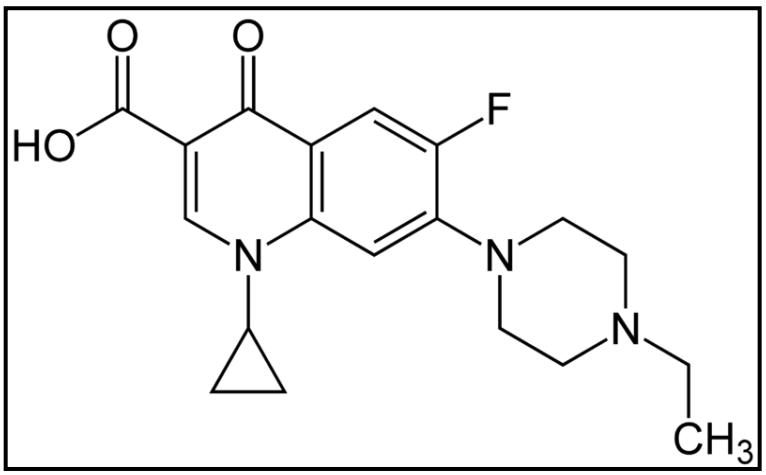

Fig. 5. Chemical structure of enrofloxacin

Antibiotic extraction:

1. Five gram of muscle/liver/kidney sample was blended in a high speed blender. Acetonitrile-0.1 M sodium hydroxide (10:1, *v/v;* 7 ml) was added to blended muscle (5 g) in a centrifuge tube.

2. After tissue dispersion, the tube was placed in an ultrasonic bath for 10 min and then centrifuged at 3200 g for 10 min at 4ºC. Supernatant was collected in a glass test tube and evaporated in contact with cold stream to dryness.

3. The residue was dissolved in dipotassium hydrogen phosphate buffer (0.05 M, pH 7.4; 7 ml) and hexane (4 ml) was gently added.

4. The tube was placed in a rotary shaker at 30 rpm for 2 min and was then centrifuged at 3200 g for 10 min. The hexane fraction was discarded.

Solid-phase extraction was performed with a Supelco system. The extract was deposited in a barrel connected to a Sep-Pak C8 SPE cartridge (3 mL, 500 rag; Waters) previously conditioned with methanol (5 ml) then dipotassium hydrogen phosphate (0.05 M, pH 7.4; 5 ml). The cartridge was washed with methanol-0.1 M ammonia solution (75:25, *v/v;* 2 ml) and eluted with methanol- 1 M ammonia solution (75:25, *v/v;* 6 ml). The eluent was evaporated to dryness and the residue was dissolved in 50 µl of methanol (Juhel-Gaugain and Abjean, 1998).

Preparation of silica plates:

Silica gel 60 F_{254} TLC plates (Merck, Germany), were activated at 60°C for 30 minutes before use to limit diffusion of the bands (Boyer, 1993).

Standard preparation

Accurately weighed 2.5 mg of enrofloxacin powder was transferred to a volumetric flask of 25 ml, dissolved and diluted to the mark with methanol to obtain a standard stock solution of enrofloxacin (0.1 mg/ml) (Thangadu *et al.,* 2002). Stock solutions were stored at 4ºC for about 1 month.

Sample application

The standard (2-10µl) and samples were spotted on TLC plates in the form of narrow bands of length 6 mm with 8 mm distance from bottom and 15 mm from left margin. The samples were applied under continuous drying stream of nitrogen gas at constant application rate of 150 nl/s.

Mobile phase and migration

Plates were developed using mobile phase consisting of butanol – ethanol – ammonia (8 : 2 : 4.4). Linear ascending development was carried out in 10 X 10 cm twin trough chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 15 minutes. Ten milliliters of the mobile phase (5 ml in trough containing the plate and 5 ml in other trough) was used for each development and allowed to migrate a distance of 90 mm. After development, the TLC plates were dried completely.

Densitometric analysis and quantitation procedure

Densitometric scanning was performed on camag TLC scanner 4 in absorbance mode and operated by winCATS planar chromatography version 1.3.4. The source of radiation utilized was deuterium lamp. The spots were analyzed at a wavelength of 280 nm. The slit dimension used in the analysis was length and width of 5 mm and 0.30 mm respectively with a scanning rate of 20 mm/sec. These are selected as recommended by the CAMAG TLC scanner 4 manual. It covers 70 – 90% of the application band length, which in the present case is 6 mm. The monochromator band width was set at 20 nm. Concentration of compound chromatographed were

determined from the intensity of diffusely reflected light and evaluated as peak area against concentrations using linear regression equation.

3. AMOXICILLIN

Amoxicillin is penicillinase-susceptible semi-synthetic penicillin. This drug is acid-stable and designed for oral use. It is rapidly and completely absorbed from the gastro-intestinal tract. It is active against respiratory tract infections caused by *Streptococcus pneumoniae*, *H. influenza, Streptococcus pyogenes*, urinary tract infection caused by *E. coli,* Streptococcus species, Proteus species; meningitis caused by *H. influenzae*, *Streptococcus pneumoniae, N. meningitis*: typhoid fever caused by Salmonella species; bacillary dysentery caused by Shigella species.

Physico-chemical properties of amoxicillin

Chemical Name **:** α-amino-p-hydroxy benzyl penicillin

Molecular Weight **:** 365.41

Half-life **:** 61.3 minutes

Excretion **:** Renal

Solubility (mg/ml) **:**

Water	:	4.0 (0.4%)
Methanol	:	7.5 (0.75%)
Absolute Ethanol	:	3.4 (0.34%)

Insoluble in hexane, benzene, ethyl acetate, acetonitrile and dichloromethane.

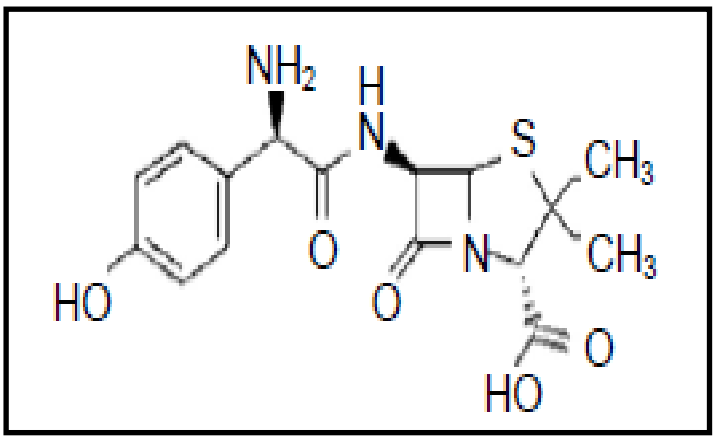

Fig. 6: Chemical structure of Amoxicillin

Antibiotics extraction:

Five gram of each sample was mixed with 5 ml ethanol (96%), crushed and squeezed finely in a Chinese mortar. The solution was transferred to 15 ml falcon centrifuge tubes and centrifuged at 7000 rpm for 10 minutes. The clear supernatant was transferred to fresh glass test tubes and evaporated in contact with cold stream. After drying the deposits were resolved in 0.2 ml methanol. The samples will be ready to point on silica plates (Tajick *et al.,* 2002).

Preparation of silica plates:

Silica gel 60 F_{254} TLC plates (Merck, Germany) with 0.25 mm thickness (Merck, Germany), were activated at 110°C for 30 minutes in hot air owen before use (Boyer, 1993).

Standard preparation

Standards was prepared by dissolving 0.5 gm of amoxicillin powder (pure technical grade) in 10 ml methanol (Thangadu *et al.,* 2002).

Sample application

The standard (2-10µl) and samples were spotted on TLC plates with the help of calibrated Hamilton syringe in the form of narrow bands of length 6 mm with 8 mm distance from bottom and 15 mm from left margin. The samples were applied under continuous drying stream of nitrogen gas at constant application rate of 150 nl/s.

Mobile phase and migration

Plates were developed using mobile phase consisting of butanol– water–dimethylsulfoxide–acetic acid (60 : 15 : 20 : 5). Linear ascending development was carried out in 10 X 10 cm twin trough chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 20 min at 25 – 30^{o}c. Ten milliliters of the mobile phase (5 ml in trough containing the plate and 5 ml in other trough) was used for each development and allowed to migrate a distance of 90 mm. After development, the TLC plates were dried completely.

Densitometric analysis and quantitation procedure

Densitometric scanning was performed on camag TLC scanner 4 in absorbance mode and operated by winCATS planar chromatography version 1.3.4. The source of radiation utilized was deuterium lamp. The spots were analyzed at a wavelength of 242 nm. The slit dimension used in the analysis was length and width of 5 mm and 0.30 mm respectively with a scanning rate of 20 mm/sec. These are selected as recommended by the CAMAG TLC scanner 4 manual. It covers 70 – 90% of the application band length, which in the present case is 6 mm. The monochromator band width was set at 20 nm. Concentration of compound chromatographed were determined from the intensity of diffusely reflected light and evaluated as peak area against concentrations using linear regression equation.

The separated band on the silica gel plate were visualized by spraying ninhydrin reagent (dissolve 0.1 gm of ninhydrin in 50 ml ethanol and add 10 ml glacial acetic acid) with the help of TLC sprayer (Camag, Switzerland) followed by heating at 110^{o}C for 10 minutes for best colour development.

Determination of effect of boiling on antibiotics residues in edible tissues of broiler chicken

Three different antibiotics from 3 different major antibiotic classes, including tetracyclines (oxytetracycline), quinolones (enrofloxacin) and beta-lactams (amoxicillin) were evaluated for thermal stability. The effect of boiling on oxytetracycline, enrofloxacin and amoxicillin residues in chicken muscle, liver and kidney samples was determined by comparing the mean diameter of inhibition zones around raw and cooked samples. Mean diameter of inhibition zones of 36 positive samples consisting of muscle, liver and kidney 12 each were recorded. The diameter of the inhibition zones was measured with a precision of 0.1 mm using a zone inhibition reader. The positive raw samples were selected for cooking process (boiling) and the test for cooked samples was performed just like raw samples after complete cooking of them. After boiling, the diameter for inhibition zones was again recorded.

Table 4: Design of experiment for determining the effect of boiling on antibiotic residues

Group	Experimental group	Tissue Samples	Number of samples	Total Number of samples
G_1	Raw sample	Muscle	12	36
		Liver	12	
		kidney	12	
G_2	Boiled sample	Muscle	12	36
		Liver	12	
		kidney	12	

Cooking operation (boiling):

Ten gram samples were placed into a strainer, immersed in water bath preheated to 100°C. These samples were then boiled in water bath, 9 min for liver and kidney samples and 24 min for muscle samples. After boiling, the diameter for inhibition zones was again recorded (Javadi *et al.*, 2011).

4. RESULTS

SURVEILLANCE STUDY

The Surveillance study was conducted on the basis of questionnaire (Passive surveillance) and personal Interviews (Active surveillance) of poultry farmers and workers. Data on the manner of using antibiotics was obtained by means of questionnaire from the poultry farmers (n = 50). The results showed that supplementation of antibiotics in both drinking water and feed was 4 percent (n = 2), and 88 percent (n = 44) poultry farmers did not consult the veterinarian for addition of antibiotics in the feed. Majority of poultry farmers (94 percent (n=47) were unaware of rules and regulations for the use of antibiotics in relation to public health. the results of surveillance study has been summarized in Tables 5 and 6.

Table 5: Results of questionnaire survey responded by the poultry farmers (n=50)

S.No.	Question	Yes	No	Some times
1.	Antibiotics addition to the feed	2	40	8
2.	Addition of antibiotics in both drinking water and food	2	40	8
3.	Additions under supervision of veterinarian	2	42	6
4.	Consultation of veterinarian	4	44	2
5.	Presence of drugs in poultry farm administer by owner in emergencies	37	10	3
6.	Consideration of withdrawal period	12	5	33
7.	Knowledge of the owners about administration of antibiotics in poultry.	50	0	0
8.	Knowledge of rules and regulation for the use of antibiotic in poultry production	3	47	0
9.	Knowledge about public health importance of antibiotic residues	5	45	0

Table 6: Commonly used antibiotics in poultry farms of Jabalpur district

S. No.	Name of the Antibiotic	S. No.	Name of the Antibiotic
1.	Oxytetracycline	2.	Enrofloxacin
3.	chlortetracycline	4.	Erythromycin
5.	Levofloxacin	6.	Amoxicillin
7.	Ciprofloxacin	8.	Gentamicin
9.	Ampicillin	10.	Cefalexin
11.	Lincomycin	12.	Doxycycline
13.	Ceftiofur	14.	Neomycin
15	Bacitracin	16.	Tylosin
17.	Chloramphenicol	18.	Tetracycline

Short listing of antibiotics for residues analysis

On the basis of surveillance studies pertaining to use of antibiotics in poultry farms located in and around Jabalpur, the following antibiotics were short-listed for residue analysis study.

- **Oxytetracycline**
- **Enrofloxacin**
- **Amoxicillin**

Identification of target areas for antibiotic residues in jabalpur

The following slaughter houses/ poultry farms were undertaken for the study

1. Sale counter, Veterinary College Jabalpur
2. Government slaughter house, Jabalpur
3. Military slaughter house
4. Joy poultry farm
5. Ansari poultry farm

Results of Microbial Growth Inhibition Assay

The presence of antibiotics residues in chicken meat samples is determined by the formation of growth inhibition zones of test microorganisms around the disc. The results of the microbiological assays of each sample were recorded as zones of inhibition in millimeters (mm). The diameter of the inhibitory zone was measured with a precision of 0.1 mm using a zone inhibition reader. A positive raw sample was indicated by a complete inhibition of growth in an annular zone measuring not less than 2 mm wide around the disc. Less than 2 mm of inhibitory zone indicated negative result (Myllyniemi *et al.,* 2001).

During a four month period (January 2012 to April 2012) 180 chicken meat samples were collected from various target areas. In this study, muscles, liver and kidney sample (60 each) were subjected to antibiotic residues screening tests. Four test bacteria namely, *Staphylococcus aureus* (MTCC 1144)*, Streptococcus pyogens* (MTCC 442)*, Escherichia coli* (MTCC 723) and *Bacillus cereus* (MTCC 1272) were used. Each sample was assayed in duplicate and the results were averaged. Among the four bacterial culture used, *Escerichia coli* was inhibited in 67 samples (37.22 percent) followed by 55 samples (30.55 percent) of *Staphylococcus aureus*, 46 samples (25.55 percent) of *Bacillus cereus* and *Streptococcus pyogens* 42 samples (23.33 percent). The results of the microbial growth inhibition assay are shown in Table 7 and 8 and Fig. 7 and 8.

Table 7: Sensitivity of test microorganisms against raw chicken meat samples.

Test microorganism	Total samples	Samples showing inhibition zones	Percentage of samples showing inhibition zones
Staphylococcus aureus	180	55	30.55
Streptococcus pyogens	180	42	23.33
Escherichia coli	180	67	37.22
Bacillus cereus	180	46	25.55

Out of 60 kidney samples tested, 35 (58.33 percent) were found positive. While, out of 60 liver samples tested, 22 (36.66 percent) were found positive. Out of 60 muscle samples tested, 19 (31.66%) were found positive.

Table 8: Sensitivity of test microorganisms against chicken muscle, liver and kidney samples.

Target organ	Total no. of samples	Samples showing positive results	Percentage of samples showing bacterial inhibition
Muscle	60	16	26.66
Liver	60	22	36.66
kidney	60	35	58.33

QUANTITATIVE HPTLC ANALYSIS OF OXYTETRACYCLINE IN CHICKEN MEAT SAMPLES

The HPTLC method for analysis of oxytetracycline was developed using the solvent system comprising of chloroform – methanol – 5% aq. Na_2EDTA (65 : 20 : 5). This mobile phase exhibited good separation of oxytetracycline from its matrix with a mean Retardation factor (R_F value) of 0.17 (Plate 1). It was also observed that chamber saturation time and solvent migration distance are crucial in chromatographic separation as chamber saturation time of less than 15 min and solvent migration distances greater than 80 mm resulted in diffusion of analyte spot. These chromatographic conditions produced a well defined compact spot of oxytetracycline with optimum migration (Plate 2). It also gave a good resolution of oxytetracycline from various components of chicken meat.

Under the experimental condition employed, the lowest amount of oxytetracycline that could be detected (LOD) was 1.668 µg/spot. However, the lowest amount of oxytetracycline that could be quantified (LOQ) was 5.055 µg/spot, with a standard deviation of 5.49 percent.

Table 9: HPTLC analysis of oxytetracycline in chicken meat samples

S.No.	Parameters	Oxytetracycline
1.	Stationary Phase	Merck TLC plates silica gel 60 F 254 (10x10 cm)
2.	Mobile Phase	Chloroform – Methanol – 5% aq. Na_2EDTA (65 : 20 : 5)
3.	Retardation factor (R_F value)	0.17
4.	Lamda (Λ) max	360 nm
5.	Correlation coefficient	0.99350
6.	Standard deviation	5.49 percent
7.	Recovery percentage	73-94
8.	Limit of Detection (LOD)	1.668 µg
9.	Limit of Quantification (LOQ)	5.055 µg

Mean residue concentration of oxytetracycline in muscle, liver and kidney samples of target areas

Mean residue concentration of oxytetracycline in muscle, liver and kidney samples were analyzed by HPTLC in different target areas of Jabalpur district. Total 180 chicken meat samples were analyzed including 36 samples each from Sale counter, Veterinary College, Government slaughter house, Military slaughter house, Joy poultry farm and Ansari poultry farm. Among the 180 samples, 23 (12.78 per cent) were found positive for oxytetracycline residues. Out of 60 kidney samples tested, 9 (15.0 per cent) were found positive for oxytetracycline residue. While, out of 60 liver samples tested, 8 (13.34 per cent) were found positive. Out of 60 muscle samples tested, 6 (10.00 per cent) were found positive.

Out of the 24 positive samples, 5 (20.83 per cent) had oxytetracycline residue at violative levels while 19 (79.17 per cent) had residue below the WHO / FAO recommended MRLs for oxytetracycline in chicken muscle, liver and kidney samples (Fig. 9).

Mean residue concentration of oxytetracycline in muscle, liver and kidney samples of Sale counter, Veterinary college were 11.56±0.0, 48.75±0.0 and 126.62±0.0 μg/kg, respectively. The Mean oxytetracycline concentrations in muscle, liver and kidney samples collected from Government slaughter house were 90.5±5.78, 230.8±74.3 and 301.21±91.36 μg/kg, respectively. Samples collected from Military slaughter house contain 127.03±8.61, 127.03±8.61 and 530.67±89.11 μg/kg oxytetracycline in muscle, liver and kidney samples, respectively. Mean residue concentration of oxytetracycline in muscle, liver and kidney samples of Joy poultry farm were 67.91±0.0, 103.61±0.0 and 141.2±97.96 μg/kg, respectively. Oxytetracycline residues in the muscle samples collected from Ansari poultry farm were found below the detection limit. However, the Mean oxytetracycline concentrations in liver and kidney samples were 42.04±17.12 and 168.19±7.99μg/kg, respectively. The comparison of mean residue concentration of oxytetracycline between groups has been summarized in Table 10 and Fig. 10.

Table 10: Mean residue concentration of oxytetracycline (μg/kg) in chicken muscle, liver and kidney samples of different target areas.

Maximum residual limit of oxytetracycline in muscle, liver and kidney of chicken by FDA: 100μg/kg=100ppb, 300μg/kg=300ppb and 600μg/kg=600ppb respectively			
Target area	**Tissue sample**		
	Muscle	**Liver**	**Kidney**
Sale counter, Veterinary College, Jabalpur	11.56±0.0	48.75±0.0	126.62±0.0
Government slaughter house, Jabalpur	90.5±5.78	230.8±74.3	301.21±91.36
Military slaughter house, Jabalpur	127.03±8.61	320.21±42.64	530.67±89.11
Joy poultry farm, Jabalpur	67.91±0.0	103.61±0.0	141.2±97.96
Ansari poultry farm Jabalpur	0.0±0.0	42.04±17.12	168.19±7.99

QUANTITATIVE HPTLC ANALYSIS OF ENROFLOXACIN IN CHICKEN MEAT SAMPLES

The HPTLC method for analysis of enrofloxacin was developed using mobile phase comprising of Butanol : Ethanol : Ammonia (8 : 2 : 4.4). This solvent system shown good separation of enrofloxacin from its matrix with a mean Retardation factor (R_F value) of 0.37 (Plate 5). The chamber was allowed to be saturated for 20 min and solvent front distance was 90 mm. These chromatographic conditions produced a well defined compact spot of enrofloxacin with optimum migration (Plate 6). It also gave a good resolution of enrofloxacin from various components of chicken meat.

This method was found to be linear in a concentration range of 100-600 ng/spot, with respect to peak area. Under the experimental condition employed, the lowest amount of enrofloxacin detected (LOD) was found to be 101.203 ng/spot and the lowest amount of enrofloxacin that could be quantified was found to be 306.67 ng/spot, with a standard deviation of 3.45 percent.

Table 11: HPTLC analysis of enrofloxacin in chicken meat samples

S.No.	Parameters	Enrofloxacin
1.	Stationary Phase	Merck TLC plates silica gel 60 F 254 (10x10 cm)
2.	Mobile Phase	Butanol : Ethanol : Ammonia (8 : 2 : 4.4)
3.	Retardation factor (R_F value)	0.37
4.	λ max	280 nm
5.	correlation coefficient	0.99389
6.	Standard deviation	3.45 percent
7.	Recovery percentage	78-97
8.	Limit of Detection (LOD)	101.203 ng
9.	Limit of Quantification (LOQ)	306.67 ng

Mean residue concentration of enrofloxacin in muscle, liver and kidney samples of target areas

Mean residue concentration of enrofloxacin in muscle, liver and kidney samples were analyzed by HPTLC in different target areas of Jabalpur district. Total 180 chicken meat samples were analyzed including 36 samples each from Sale counter, Veterinary College, Government slaughter house, Military slaughter house, Joy poultry farm and Ansari poultry farm. Among the 180 samples, 37 (20.55 percent) were found positive for enrofloxacin residues. Out of 60 kidney samples tested, 16 (26.67 percent) were found positive for enrofloxacin residue. While, out of 60 liver samples tested, 12 (20.0 percent) were found to be positive. Out of 60 muscle samples tested, 9 (15.0 percent) were found positive.

Out of the 37 positive samples, 9 (24.32) percent had enrofloxacin residue at violative levels while 28 (75.68 percent) had residue below the WHO / FAO recommended MRLs for enrofloxacin in chicken muscle, liver and kidney samples (Fig. 11).

Mean residue concentration of enrofloxacin in muscle, liver and kidney samples of Sale counter, veterinary college were 15.49±4.41, 35.35±16.63, 83.49±24.60 ppb respectively. The Mean enrofloxacin concentrations in muscle, liver and kidney samples collected from Government slaughter were 64.4±49.16, 172.66±54.12 and 247.91±61.63μg/kg respectively. Samples collected from Military slaughter house contain 13.85±0.0, 178.85±46.59 and 113.96±57.67μg/kg enrofloxacin in muscle, liver and kidney samples respectively. Mean residue concentration of enrofloxacin in muscle, liver and kidney samples of Joy poultry farm were 75.22±46.54, 58.24±23.86 and 244.36±79.26μg/kg respectively. The Mean enrofloxacin concentrations in muscle, liver and kidney samples collected from Ansari poultry farm were 68.04±36.12, 170.64±34.88 and 229.56±64.44 respectively.

Table 12: Mean residue concentration of enrofloxacin in chicken muscle, liver and kidney samples of different target areas.

Maximum residual limit of enrofloxacin in muscle, liver and kidney of chicken by FDA: 100μg/kg=100ppb, 200μg/kg=200ppb and, 300μg/kg=300ppb respectively			
Target area	**Tissue sample**		
	Muscle	**Liver**	**Kidney**
Sale counter, Veterinary College, Jabalpur	15.49±4.41	35.35±16.63	83.49±24.60
Government slaughter house, Jabalpur	64.4±49.16	172.66±54.12	247.91±61.63
Military slaughter house, Jabalpur	13.85±0.0	178.85±46.59	113.96±57.67
Joy poultry farm, Jabalpur	75.22±46.54	58.24±23.86	244.36±79.26
Ansari poultry farm Jabalpur	68.04±36.12	170.64±34.88	229.56±64.44

QUANTITATIVE HPTLC ANALYSIS OF AMOXYCILLIN IN CHICKEN MEAT SAMPLES

The HPTLC method for analysis of amoxicillin was developed using the solvent system comprising of Butanol – Water – Dimethylsulfoxide – Acetic acid (60 : 15 : 20 : 5). This mobile phase gives gave good separation of amoxicillin from its matrix with a mean Retardation factor (R_F value) of 0.53 (Plate 9). The chamber is allowed to be saturated for 15 min and solvent front distance was 80 mm. These chromatographic conditions produced a well defined compact spot of amoxicillin with optimum migration (Plate 10). It also gave a good resolution of amoxicillin from various components of chicken meat.

This method was found to be linear in a concentration range of 2-10 µg/spot, with respect to peak area. Under the experimental condition employed, the lowest amount of amoxicillin that could be detected (LOD) was found to be 0.449 µg/spot and the lowest amount of amoxicillin that could be quantified was found to be 1.363 µg/spot, with a standard deviation of 1.74 percent.

Table 13: HPTLC analysis of amoxicillin in chicken meat samples

S.No.	Parameters	Enrofloxacin
1.	Stationary Phase	Merck TLC plates silica gel 60 F 254 (10x10 cm)
2.	Mobile Phase	Butanol – Water – Dimethylsulfoxide – Acetic acid (60 : 15 : 20 : 5)
3.	Retardation factor (R_F value)	0.53
4.	λ max	242 nm
5.	Regression coefficient	0.99897
6.	Standard deviation	1.74 percent
7.	Recovery percentage	81-90
8.	Limit of Detection (LOD)	0.449 µg
9.	Limit of Quantification (LOQ)	1.363 µg

Mean residue concentration of amoxicillin in muscle, liver and kidney samples of target areas

Mean residue concentration of amoxicillin in muscle, liver and kidney samples were analyzed by HPTLC in different target areas of Jabalpur district. Total 180 chicken meat samples were analyzed including 36 samples each from Sale counter, Veterinary College, Government slaughter house, Military slaughter house, Joy poultry farm and Ansari poultry farm. Among the 180 samples, 19 (10.55 percent) were found positive for amoxicillin residues. Out of 60 kidney samples tested, 7 (11.67 percent) were found positive for amoxicillin residue. While, out of 60 liver samples tested, 7 (11.67 percent) were found to be positive. Out of 60 muscle samples tested, 5 (8.33 percent) were found to be positive.

Out of the 180 chicken samples analyzed during this study, 19 (10.55 percent) samples had detectable levels of amoxicillin residues while remaining 156 samples (45.6%) were found free from detectable amoxicillin residues. Out of the 19 positive samples, 6 (3.34 percent) had amoxicillin residues levels higher than the WHO / FAO recommended MRLs levels for amoxicillin in chicken muscle, liver and kidney samples (Fig. 13).

Among the 36 samples collected from sale counter, veterinary college Jabalpur no any sample was detected positive for amoxicillin residue. Samples collected from Government slaughter house showed mean residue concentration of amoxicillin 25.65±0.0 ppb, 60.22±0.0 ppb and 63.98±0.0 ppb in, liver and kidney samples respectively. Amoxicillin was not detected in the muscle samples of Military slaughter house however, the mean residue concentration in liver and kidney was 27.65±0.0 and 39.45±0.0 ppb respectively. Mean residue concentration of amoxicillin in muscle, liver and kidney samples of Joy poultry farm was 4.92±0.0, 31.04±0.0 and 49.28±0.0 ppb respectively. However, 31.77±19.31, 26.11±10.44 and 55.87±15.70 ppb were the mean residue concentration of amoxicillin in muscle, liver and kidney samples respectively.

Table 14: Mean residue concentration of amoxicillin in chicken muscle, liver and kidney samples of different target areas.

Maximum residual limit of amoxicillin in muscle, liver and kidney of chicken by FDA: 50μg/kg=50ppb			
Target area	**Tissue sample**		
	Muscle	**Liver**	**Kidney**
Sale counter, Veterinary College, Jabalpur	0.00	0.00	0.00
Government slaughter house, Jabalpur	25.65	60.22	63.98
Military slaughter house, Jabalpur	0.00	27.65	39.45
Joy poultry farm, Jabalpur	4.92	31.04	49.28
Ansari poultry farm Jabalpur	31.77±19.31	26.11±10.44	55.87±15.70

Determination of effect of boiling on antibiotics residues in edible tissues of broiler chicken

The effect of boiling on oxytetracycline enrofloxacin and amoxicillin residues in chicken muscle, liver and kidney samples were determined by comparing the mean diameter of inhibition zones around raw and cooked samples. All the tissue samples showed reduction in their inhibition zone diameter after boiling.

However, in order to make the results more meaningful and readily intelligible, the tables record the percentage reduction in inhibition zone diameters after cooking.

Effect of boiling on oxytetracycline residues

The mean inhibition zone diameter of raw muscle, liver and kidney samples positive for oxytetracycline residues were 9.17±1.58, 11.75±2.41 and 13.6±2.23 mm respectively. After boiling the mean diameter of zone of inhibition reduced to 4.23±0.64, 5.98±1.21 and 7.22±1.18 mm for boiled muscle, liver and kidney samples respectively, showing a percentage reduction of 53.87, 49.11 and 46.91 in inhibition zone diameter. The comparison of mean diameter of inhibition zones of raw and boiled sample has been summarized in table 15 and Fig. 15.

Table 15: Effect of boiling on oxytetracycline residues (inhibition zone) in muscle, liver and kidney samples of chicken

Tissue sample	Raw samples	Boiled samples	Percentage reduction
Muscle	9.17±1.58	4.23±0.64	53.87
Liver	11.75±2.41	5.98±1.21	49.11
Kidney	13.6±2.23	7.22±1.18	46.91

Effect of boiling on enrofloxacin residues

The mean diameter of inhibition zone around raw and cooked meat sample containing enrofloxacin residue is shown in table 16 and Fig. 16. The mean inhibition zone diameter of raw muscle, liver and kidney samples positive for enrofloxacin residues were 6.67±0.99, 9.58±1.06 and 12.25±1.33 mm respectively. After boiling the mean diameter of zone of inhibition reduced to 6.35±0.91, 9.02±0.96 and 11.35±1.21 mm for boiled muscle, liver and kidney samples respectively, showing a percentage reduction of 4.80, 5.84 and 7.35 in inhibition zone diameter.

Table 16: Effect of boiling on enrofloxacin residues in muscle, liver and kidney samples of chicken

Tissue sample	Raw samples	Boiled samples	Percentage reduction
Muscle	6.67±0.99	6.35±0.91	4.80
Liver	9.58±1.06	9.02±0.96	5.84
Kidney	12.25±1.33	11.35±1.21	7.35

Effect of boiling on amoxycillin residues

The mean inhibition zone diameter of raw muscle, liver and kidney samples positive for amoxicillin residues were 6.5±2.10, 8.83±1.58 and 13.83±1.64 mm respectively. After boiling the mean diameter of zone of inhibition reduced to 5.1±1.93, 7.2±1.42 and 11.4±1.54 mm for boiled muscle, liver and kidney samples respectively, showing a percentage reduction of 21.54, 18.46 and 17.57 in inhibition zone diameter.

Table 17: Effect of boiling on amoxycillin residues (inhibition zone) in muscle, liver and kidney samples of chicken

Tissue sample	Raw samples	Boiled samples	Percentage reduction
Muscle	6.5±2.10	5.1±1.93	21.54
Liver	8.83±1.58	7.2±1.42	18.46
Kidney	13.83±1.64	11.4±1.54	17.57

5. DISCUSSION

Antibiotics may be the most abused and the most useful class of drugs available. They are used to treat infectious diseases, which are one of the most common problems seen in animals as well as humans. Although, primary bacterial infections do occur, secondary infections due to poor husbandry practices and immunosuppression are more common in poultry. Poor husbandry practices can expose birds to large numbers of potentially pathogenic organisms from environmental sources and other birds. Malnutrition, stress, medications, or concurrent disease may results in immunosuppression, leading to increased susceptibility to potential pathogens. Infectious disease management in poultry thus involves frequent use of antibiotics.

To ensure enough food production to feed the enlarging world population, animal husbandry practices are pushed towards a more intensive production. Intensification of animal production leads to an increase of bacterial infections and consequently a higher therapeutic and even prophylactic use of antibiotics. The excessive use of antibiotics may result in the presence of residues of these substances or their metabolites in food from animal origin.

In order to protect the consumer, Maximum Residue Limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin, were set by "Codex Alimentarius Commission" which is jointly set up by Food and Agricultural Organization (FAO), World Health Organization (WHO) and the European Union, on the basis of scientific assessment of the safety of those substances. Protection of public health against possible harmful effects of antibiotic residues is a major concern. Therefore, a large number of samples need to be analysed for injudicious use of antibiotics. For the determination of residues of antibiotics in food, microbial screening methods are widely used because of their cost-effectiveness. By preference screening methods should possess the following characteristics: high throughput, short analysis time, ease of use, low cost, cheap instrumentation, minimal sample pretreatment, possibility of automation and detection of the analyte or family of analytes at the level of interest.

Till present, no single rapid screening method is able to detect all antibiotics at their respective MRL. Even more, no single microbiological test can screen meat for all antibiotics and chemotherapeutics with a MRL at the regulatory level. To ensure food safety of chicken meat from antibiotic residues, combinations of screening tests are needed, or even screening tests may be supplemented by other types of methods like HPLC, HPTLC and LC-MS in order to cover all MRLs. A bridging study was therefore, conducted to establish the correlation between microbial inhibition (MI) method and liquid chromatographic method for analysis of antibiotic residues in chicken meat.

A surveillance study was conducted pertaining to use of antibiotic in various poultry farms located in and around Jabalpur district. The study was conducted on the basis of questionnaire (Passive surveillance) and personal interviews (Active surveillance) of poultry farmers and workers. Questionnaire survey revealed that the supplementation of antibiotics in feed and water is not done under the supervision of veterinarian in 84 per cent poultry farms. Therefore, the dose regimen and administration of antibiotics was left with the poultry farmers. Weight of animal was not considered when describing doses in poultry which lead to over-dosing or sub dosing of birds. Similarly, Sawant *et al*. (2005) found the same results during his survey on antibiotic usage in Pennsylvania.

The most of the antibiotics used in the poultry farms were the broad spectrum antibiotics (96 per cent), without use of diagnostic methods for microbial assay. Therefore, veterinarian and farmers are depended only on the tentative diagnosis and using umbrella treatment. Poultry farmers mostly slaughter their animals during treatment with antibiotics (32 per cent) or before the withdrawal period (66 per cent). This leads to the high percentage of antibiotics residues in chicken meat. The similar findings were reported by Ibrahim *et al.* (2010) who found most of veterinary drugs including antibiotics were used as non specific treatments.

The surveillance data revealed that most of poultry farmers are aware of antibiotic toxicity in terms of residual harmful effects in poultry meat used for human consumption. However, these farmers do not follow the

recommended antibiotic withdrawal period after the use of antibiotic. The findings are in close agreement to those of Al-Ghamdi *et al.* (2000) and Salehzadeh *et al.* (2006). The non-compliance to withdrawal period by farmers could be associated with many reasons including fear of economic losses. Most of the poultry keepers are subsistence farmers and since there is frequent occurrence of diseases which need regular therapeutic uses of drugs in poultry. The implementation of withdrawal period may cause delay in sale of birds leading to huge losses to the poultry farmers. The other reason could be lack of awareness to farmers on the possible side effects of antimicrobials and other drugs to animals and humans.

The data on use of various antibiotics were further analyzed for short listing of most commonly used three antibiotics for determination of antibiotic residues in muscle, liver and kidney samples of chickens. The data collected from various poultry farmers clearly indicated that the antibiotics namely oxytetracycline, enrofloxacin and amoxicillin, were most commonly used in poultry farms. These findings are similar to the observation of Nonga *et al.,* (2009), Naeem *et al*. (2006) and Al-Ghamdi *et al.* (2000) who also found higher usage of tetracylines and fluoroquinolones in chickens. Therefore, oxytetracycline, enrofloxacin and amoxicillin were shortlisted for further investigations.

Microbial assay methods are qualitative screening test, which are commonly used to detect antibiotic residues. The advantages of these tests are quite simple, relatively inexpensive, easy to use, sensitive, reliable and can be efficiently adopted by laboratory staff. The other advantages are the option to analyze a large number of samples simultaneously and the relatively short period of time needed for preparation of samples as no purification procedures are required. They cannot be used to identify individual antibiotics and positive result should be confirmed with chemical or physical methods.

Among the four bacterial culture used *Escerichia coli* was inhibited in 67 samples (37.22 per cent) followed by *Staphylococcus aureus* in 55 samples (30.55 per cent), *Bacillus cereus* in 46 samples (25.55 per cent) and *Streptococcus pyogens* in 42 samples (23.33 per cent). These results are

in accordance with Ellerbroek (1991) and Petrovic et al. (2006) who found that the sensitivity of *E. coli* towards enrofloxacin was 3–30 times more as compared to other test microorganisms.

Among the three different tissue sample kidney showed the highest percentage of microbial growth inhibition followed by liver and muscle. Out of 60 kidney samples tested, 35 (58.33 per cent) were found positive. While, out of 60 liver samples tested, 22 (36.66 per cent) were found positive. Out of 60 muscle samples tested, 19 (31.66%) were detected positive. The findings substantiate the work of Pavlov *et al.* (2005), Alla *et al.,* (2011) and Salehzadeh *et al.* (2007). This may be explained by the fact that the excretion of most of the antibiotics such as tetracyclines, animoglycosides, chloramphinicol and quinolones occur through the kidneys.

The chromatographic techniques are the most efficient analytical tool for detection of antibiotic residues in minute quantities. The chromatographic methods used for the antibiotic residue analysis includes Thin Layer Chromatography (TLC), Gas Chromatography (GC), Liquid Chromatography (LC), High Performance Thin Layer Chromatography (HPTLC), and High Performance Liquid Chromatography (HPLC).

HPTLC methods discriminate well between analytes and closely related substances based on the use of particular extraction procedures and separation methods and thus, offer good performance for the identification and quantification of antibiotics in foods. The possibility of automation (injection, development, detection) and computer-control makes HPTLC an adequate instrument as a quantitative technique In addition, HPTLC has the great potential of coupling to mass spectrometry in case that a final confirmation of a given suspicious sample be required.

HPTLC allows the qualitative and quantitative detection of multi-residues in meat. Reported uses of HPTLC applied to meat include the detection of residues like clenbuterol and other agonists (Degroodt *et al.,* 1991), nitroimidazol (Gaugain and Abjean, 1996) and sulfonamides (Van Poucke *et al.,* 1991) and thyreostatic drugs (De Wasch *et al.,* 1998). The plates are sprayed with an appropriate chromogenic reagent or viewed under UV light

for visualisation of compounds. Detection by fluorescence is also applied. Quantitation is achieved by measuring the relative intensity of the spot verses that of the internal standard by scanning densitometry. Modern HPTLC has been automatised at a high level.

Tetracyclines have served for decades as an important class of antibiotics in food animal health and production. As such, they have also been a source of concern for residue monitoring authorities around the world. In response to this concern the World Health Organization and the Food and Agriculture Organization (FAO 1999) joint committee on residues of some veterinary drugs in animals and foods recommended maximum residue limit (MRL) for oxytetracycline in chicken muscle (100 µg/kg), liver (300 µg/kg) and kidney (600 µg/kg).

In the present study mean residue concentration of oxytetracycline in muscle, liver and kidney samples were analyzed by HPTLC in different target areas of Jabalpur district. Total 180 chicken meat samples were analyzed including 36 samples each from Sale counter, Veterinary College, Government slaughter house, Military slaughter house, Joy poultry farm and Ansari poultry farm. As revealed by this study, 23 samples (12.78%) showed detectable levels of oxytetracycline residues. Oxytetracycline undergoes extensive entrohepatic circulation which leads to prolongation of their elimination half-lives; thus persisting in the body for a long time even after cessation of drug administration. The prevalence of oxytetracycline residue obtained in this study is lower then reported by Shareef *et al*. (2009) and Kabir *et al*. (2004), which may be because of difference in pattern of drug used at the place of study.

The mean residue concentration of oxytetracycline in muscle, liver and kidney samples were 74.25±24.19, 167.31±46.21 and 271.04±57.60 µg/kg respectively. Liver and kidney samples yielded more positive results with higher residual concentration as compared to the muscle samples. Out of 180 samples, 5 samples (2.78%) detected with residue levels above maximum residue limit (WHO 1999). The findings are in accordance with Dipeolu and Alonge (2002) and Alhendi *et al.* (2000). They also found the highest concentration of antibiotics in kidney followed by liver and muscles.

Mean residue concentration of enrofloxacin in muscle, liver and kidney samples were analyzed by HPTLC in different target areas of Jabalpur district. The mean recovery percentage of enrofloxacin was 87.5 per cent and the limit of quantification of was 0.31µg/kg. Garcia *et al.* (2004) obtained similar recovery percentage (87.7 per cent) and a slightly lower quantification level (0.1 µg/kg).

Total 180 chicken meat samples were analyzed including 36 samples each from Sale counter, Veterinary College, Government slaughter house, Military slaughter house, Joy poultry farm and Ansari poultry farm. Among the 180 samples, 37 (20.55 per cent) were found positive for enrofloxacin residues. The mean residue concentration of enrofloxacin was 51.13±15.74, 120.47±23.73 and 177.36±30.17 in muscle, liver and kidney samples, respectively. These results are in accordance with Naeem *et al.* (2006) however higher than the findings of Salehzadeh *et al.* (2007). In case of enrofloxacin residues the relatively lower concentration in liver is attributable to its primary metabolite formation and its higher kidney residue levels are due to its higher lipophylicity.

Out of the 37 positive samples, 9 (5.0 per cent) showed enrofloxacin residue at violating levels while 28 samples (75.68 per cent) had residue concentration below the WHO / FAO recommended MRLs for enrofloxacin in chicken muscle, liver and kidney samples. The results further revealed that out of 9 samples above the MRL levels including 4 (10.81 per cent) were kidney samples followed by 3 (8.11) liver sample and 2 (5.41) muscle samples. These results are in agreement with Salehzadeh *et al.* (2007) who also reported highest number of drug violations in kidney followed by liver and muscle samples.

Mean residue concentration of amoxicillin in muscle, liver and kidney samples were analyzed by HPTLC in different target areas of Jabalpur district. Total 180 chicken meat samples were analyzed including 36 samples each from Sale counter, Veterinary College, Government slaughter house, Military slaughter house, Joy poultry farm and Ansari poultry farm. Among the 180 samples, 16 (8.89 per cent) were found positive for amoxicillin residues.

The mean residue concentration of amoxicillin in muscle, liver and kidney samples were 23.53±10.13, 32.88±7.23 and 53.39±7.79 µg/kg respectively. Out of the 16 positive samples, 6 samples (3.34 per cent) had amoxicillin residues levels higher than the WHO / FAO recommended MRLs levels for amoxicillin in chicken muscle, liver and kidney samples. Singh (2010) also reported similar concentration of amoxicillin in milk samples collected from different dairy farms of Jabalpur.

In the present study, high levels of oxytetracycline, enrofloxacin and amoxicillin was detected in edible chicken tissues, including kidney and liver with higher concentrations of residues than muscle. This is in agreement with the known pharmacokinetic profiles of these drugs as these drugs are distribute widely into body tissues and are found in high concentrations in the excretory organs, especially the liver and kidney.

In this study, three most commonly used antibiotics including tetracyclines (oxytetracycline), quinolones (enrofloxacin) and beta-lactams (amoxicillin) were evaluated for thermal stability. The effect of boiling on antibiotics residues in chicken muscle, liver and kidney samples was determined by comparing the mean diameter of inhibition zones in raw and cooked samples of liver, kidney and muscle.

The mean inhibition zone diameter of raw muscle, liver and kidney samples positive for oxytetracycline residues were 9.17±1.58, 11.75±2.41 and 13.6±2.23 mm respectively. After boiling the mean diameter of zone of inhibition reduced to 4.23±0.64, 5.98±1.21 and 7.22±1.18 mm for boiled muscle, liver and kidney samples respectively, showing a percentage reduction of 53.87, 49.11 and 46.91 per cent in inhibition zone diameter. Similarly, Hassani *et al.* (2008) reported the heat labile nature of oxytetracycline.

To observe the effect of cooking, comparison between the mean inhibition zone diameters of enrofloxacin containing raw and cooked meat sample was done. The results revealed percentage reduction of 4.80, 5.84 and 7.35 per cent in inhibition zone diameter for boiled muscle, liver and kidney samples respectively. The results of our study showing the fate of drug

residues are consistent with the findings of Javadi *et al.* (2011) and Lolo *et al.* (2006). Enrofloxacin residues remained stable during heating. However, there was an apparent decrease in quinolone concentration in tissue because some amount of drug was lost by exudation into the liquid used for cooking. The results are not in accordance with Van-Egmond *et al*. (2000) who reported reduction of 68 per cent of residual activity after cooking. This variation in findings can be attributed to difference in the meat sample (pork instead of chicken meat) and higher temperature (134^{o}C instead of 100^{o}C) used for cooking.

The mean inhibition zone diameter of raw muscle, liver and kidney samples for amoxicillin residues were 6.5±2.10, 8.83±1.58 and 13.83±1.64 mm respectively. After boiling the mean diameter of zone of inhibition reduced to 5.1±1.93, 7.2±1.42 and 11.4±1.54 mm for boiled muscle, liver and kidney samples respectively, showing a reduction of 21.54, 18.46 and 17.57 per cent in inhibition zone diameter. The partial heat labile nature of amoxicillin was also demonstrated by Traub and Leonhard (1995).

It may be concluded that cooking processes cannot annihilate the total amounts of antibiotic residues but it could only decrease their amount in boiled samples as compared to raw samples. Various parameters like cooking time and cooking temperature involved during the process of cooking may also affect the antibiotic residue concentration.

6. SUMMARY CONCLUSION AND SUGGESTIONS FOR FURTHER WORK

6.1 Summary

Antibiotics are widely used in veterinary practice to control, prevent and treat infections; and to enhance animal growth and feed efficiency. The frequent and indiscriminate use of antibiotics resulted in contamination of edible poultry tissue with antibiotic residues. Protection of public health against possible harmful effects of antibiotic residues is a major concern.

In this study, surveillance work was conducted pertaining to use of antibiotics in various poultry farms located in and around Jabalpur district. The results of questionnaire survey revealed that the supplementation of antibiotics in feed and water is not done under the supervision of veterinarian in 84 per cent poultry farms and most of poultry farmers (94 per cent) were unaware of rules and regulations for the use of antibiotics in relation to public health. The information was gathered to shortlist most commonly and frequently used three antibiotics in chickens and hence oxytetracycline, enrofloxacin and amoxicillin were short listed for determination of their residual concentration in chicken meat samples.

A total of 180 poultry meat samples including muscle, liver and kidney 60 each were collected randomly from slaughtered birds of selected target areas located in and around Jabalpur. The qualitative estimation of antibiotic residues was done using microbial growth inhibition assay method however; High Performance Thin Layer Chromatography (HPTLC) was used for the quantitative detection of antibiotic residues in meat.

Among the four bacterial culture used in microbial growth inhibition assay, *Escerichia coli* was inhibited in 67 samples (37.22 per cent) followed by *Staphylococcus aureus* in 55 samples (30.55 per cent), *Bacillus cereus* in 46 samples (25.55 per cent) and *Streptococcus pyogens* in 42 samples (23.33 per cent). Out of 60 kidney samples tested, 35 (58.33 per cent) were found positive. While, out of 60 liver samples tested, 22 (36.66 per cent) were found positive. Out of 60 muscle samples tested, 19 (31.66 per cent) were found positive.

Mean residue concentration of oxytetracycline in muscle, liver and kidney samples of Sale counter, Veterinary College were 11.56±0.0, 48.75±0.0 and 126.62±0.0 μg/kg, respectively. The Mean oxytetracycline concentrations in muscle, liver and kidney samples collected from Government slaughter house were 90.5±5.78, 230.8±74.3 and 301.21±91.36 μg/kg, respectively. Samples collected from Military slaughter house contain 127.03±8.61, 127.03±8.61 and 530.67±89.11 μg/kg oxytetracycline in muscle, liver and kidney samples, respectively. Mean residue concentration of oxytetracycline in muscle, liver and kidney samples of Joy poultry farm were 67.91±0.0, 103.61±0.0 and 141.2±97.96 μg/kg, respectively. Oxytetracycline residues in the muscle samples collected from Ansari poultry farm were found below the detection limit. However, the Mean oxytetracycline concentrations in liver and kidney samples were 42.04±17.12 and 168.19±7.99μg/kg, respectively.

Mean residue concentration of enrofloxacin in muscle, liver and kidney samples of Sale counter, veterinary college were 15.49±4.41, 35.35±16.63, 83.49±24.60 ppb respectively. The Mean enrofloxacin concentrations in muscle, liver and kidney samples collected from Government slaughter were 64.4±49.16, 172.66±54.12 and 247.91±61.63μg/kg respectively. Samples collected from Military slaughter house contain 13.85±0.0, 178.85±46.59 and 113.96±57.67μg/kg enrofloxacin in muscle, liver and kidney samples respectively. Mean residue concentration of enrofloxacin in muscle, liver and kidney samples of Joy poultry farm were 75.22±46.54, 58.24±23.86 and 244.36±79.26μg/kg respectively. The Mean enrofloxacin concentrations in muscle, liver and kidney samples collected from Ansari poultry farm were 68.04±36.12, 170.64±34.88 and 229.56±64.44 respectively.

Among the 36 samples collected from Sale counter, Veterinary College Jabalpur none of the sample was detected positive for amoxicillin residue. Samples collected from Government slaughter house showed mean residue concentration 25.65±0.0 ppb, 60.22±0.0 ppb and 63.98±0.0 ppb of amoxicillin in, liver and kidney samples respectively. Amoxicillin was not detected in the muscle samples of Military slaughter house however, the

mean residue concentration in liver and kidney was 27.65±0.0 and 39.45±0.0 ppb respectively. Mean residue concentration of amoxicillin in muscle, liver and kidney samples of Joy poultry farm was 4.92±0.0, 31.04±0.0 and 49.28±0.0 ppb respectively. However, 31.77±19.31, 26.11±10.44 and 55.87±15.70 ppb were the mean residue concentration of amoxicillin in muscle, liver and kidney samples respectively.

The effect of boiling on oxytetracycline enrofloxacin and amoxicillin residues in chicken muscle, liver and kidney samples were determined by comparing the mean diameter of inhibition zones around raw and cooked samples. The mean inhibition zone diameter of raw muscle, liver and kidney samples positive for oxytetracycline residues were 9.17±1.58, 11.75±2.41 and 13.6±2.23 mm respectively. After boiling the mean diameter of zone of inhibition reduced to 4.23±0.64, 5.98±1.21 and 7.22±1.18 mm for boiled muscle, liver and kidney samples respectively, showing a percentage reduction of 53.87, 49.11 and 46.91 per cent in inhibition zone diameter.

The mean inhibition zone diameter of raw muscle, liver and kidney samples positive for enrofloxacin residues were 6.67±0.99, 9.58±1.06 and 12.25±1.33 mm respectively. After boiling the mean diameter of zone of inhibition reduced to 6.35±0.91, 9.02±0.96 and 11.35±1.21 mm for boiled muscle, liver and kidney samples respectively, percentage reduction of 4.80, 5.84 and 7.35 per cent in inhibition zone diameter.

The mean inhibition zone diameter of raw muscle, liver and kidney samples for amoxicillin residues were 6.5±2.10, 8.83±1.58 and 13.83±1.64 mm respectively. After boiling the mean diameter of zone of inhibition reduced to 5.1±1.93, 7.2±1.42 and 11.4±1.54 mm for boiled muscle, liver and kidney samples respectively, showing a reduction of 21.54, 18.46 and 17.57 per cent in inhibition zone diameter.

6.2 Conclusion

1. The surveillance study conducted at fifty poultry farms in and around Jabalpur revealed the therapeutic and growth promoting use of a wide range of antibiotics including tetracyclines, fluoroquinolones, beta-lactams and macrolide antibiotics. The three most commonly used antibiotics viz oxytetracycline, enrofloxacin and amoxycillin were short listed for experimentation.

2. Results of microbial growth inhibition assay showed that out of four test microorganisms *Escerichia coli* is inhibited by maximum number of raw chicken meat samples followed by *Staphylococcus aureus, Bacillus cereus* and *Streptococcus pyogens.*

3. Among three antibiotics, enrofloxacin was the most predominant antibiotic (20.55 percent) detected in 180 chicken meat samples, followed by oxytetracycline (13.33 per cent) and amoxycillin (10.55 per cent).

4. The concentration of three antibiotics was maximum to the extent of 58.33 per cent in kidney samples of chicken, followed by 50 per cent in liver and 38.33 per cent in muscle samples. Out of 180 samples, the residue concentration of three antibiotics in 19 samples (10.55 per cent) was found to be above the Maximum Permissible Limit.

5. Among the three antibiotics studied, oxytetracycline was largely inactivated by heat while, amoxicillin was partially heat labile and enrofloxacin was found resistant to heat.

6.3 Suggestions for Further Work

1. The study should be further extended to determine residues concentration of oxytetracycline, enrofloxacin and amoxicillin in eggs and other poultry products.

2. The study needs to explore the residues concentration of oxytetracycline, enrofloxacin and amoxicillin in muscle, liver and kidney samples of other food animals.

3. The metabolites of oxytetracycline, enrofloxacin and amoxicillin should also be determined for their residual toxicity.

REFERENCES

Aarestrup, F. M., H. C. Wegener, and P. Collignon (2008). Resistance in bacteria of the food chain: epidemiology and control strategies. *Expert Rev Anti Infect Ther*., **6**(5):733-50.

Adams H. R. (2001). Veterinary pharmacology and therapeutics. 8 ed. Iowa state press. USA. Pp:310-311.

Aerts, M. M. L., A. C. Hogenboom and U. A. Brinkman (1995). Analytical strategies for the screening of veterinary drugs and their residues in edible products. *J. Chromatogr. B: Biomed. Sci. Appl*., **667**(1):1-40.

Alali, W. Q., H. M. Scott, R. B. Harvey, B. Norby, D. B. Lawhorn and S. D. Pillai (2008). Longitudinal study of antimicrobial resistance among *Escherichia coli* isolates from integrated multi-site cohorts of humans and swine. *Appl. Environ. Microbiol.,* **74**:3672-3681.

Al-Ghamdi, M. S., Z. H. Al-Mustafa, F. El-Morsy, A. Al-Faky, I. Haider and H Essa (2000). Residues of tetracycline compounds in poultry products in the eastern province of Saudi Arabia. *Public Health,* **114:**300-304.

Alhendi, A. B., A. A. M. Homeida and E. S. Gaili (2000). Drug residues in broiler chickens fed with antibiotics in ration. *Vet. Arhiv.,* **70**:199-205.

Alla, M. B. W., T. E. Mohamed and A. E. Abdelgadir (2011). Detection of antibiotics residues in beef in Ghanawa Slaughterhouse, Khartoum State, Sudan. *African Journal of Food Science,* **5**(10):574-580.

Amjad, H., J. Iqbal and M. Naeem (2005). Analysis of Some Residual Antibiotics in Muscle, Kidney and Liver Samples of Broiler Chicken by Various Methods. *Pakistan Acad. Sci.*, **42**(4):223-231.

Argekar, A. P., S. U. Kapadia and S. V. Raj (1996). Determination of some fluoroquinolones in pharmaceutical preparations by HPTLC method. *Indian drugs,* **33**(3):107-111.

Barza, M. (2002). Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals. *Clin Infect Dis*., **34**(3):S123-5.

Bauer, A. W., W. M. M. Kieby, J. C. Shrenis and M. Turck (1966). Antibiotic susceptibity testing by a standardized single disc diffusion method. *American Journal of Clinical Pathology*, **45:**453-496.

Bharuch B. (2009). Data from IMS Health Information and Consulting Services-India. In: Laxminarayan R, editor. Courtesy of Pfizer.

Biswas, A. K., G. S. Rao, N. Kondaiah, A. S. R. Anjaneyulu, S. K. Mendiratta, R. Prasad and J. K. Malik (2007). Simple Multi-residue Method for Determination of Oxytetracycline, Tetracycline and Chlortetracycline in Export Buffalo Meat by HPLC-Photodiode Array Detector. *Journal of Food and Drug Analysis*, **15**(3):278-284.

Botsoglou, N. A. and D. J. Fletouris (2000). Drug Residues In Foods, Pharmacology, Food Safety and Analysis. Marcel Dekker, Inc. New York.

Boyer, R. F. (1993). Modern experimental biochemistry. 2nd ed. Benjamin Cummings Publishing Company Redwood City, CA., P:550.

Brynes, S.D. and M.S. Yong (1993). Harmonization of tolerances under the free trade agreement between the United States and Canada. *Proceedings of the Euro-Residue II Conference,* Veldhoven, The Netherlands, 3-5 May.

Brynes, S.D., S.F. Sundlof, A. Vilim, G. Lambert, M.S. Yong and S.Z. Fitzpatrick (1996). International harmonization of maximum residue levels for veterinary drugs via dietary intake estimates. *Proceedings of the Euro-Residue III conference,* Veldhoven, The Netherlands, 6-8 May.

Butaye, P., L. A. Devriese and F. Haesebrouck (2001). Differences in antibiotic resistance patterns of Enterococcus faecalis and Enterococcus faecium strains isolated from farm and pet animals. *Antimicorbial Agents and Chemotherapy,* **45**:1374-1378.

Caren, L., C. Rosa, G. Merce and M. Dolors-Prats (2001). Determination of macrolide antibiotics by liquid chromatography. *Journal of Chromatography A*, **910**:285–290.

Cerniglia, C. E. and S. Kotarski (1999) Evaluation of Veterinary Drug Residues in Food for Their Potential to Affect Human Intestinal Microflora. *Regulatory Toxicology and Pharmacology,* **29**:238–261.

Chafer-Pericas C., A. Maquieira and R. Puchades (2010). Fast screening methods to detect antibiotic residues in food samples. *Trends in Analytical Chemistry* **29**(9):1038-1049.

Choi, J., A. J. Yee, D. Thompson, J. Samoluk and M. Mitchel (1999). Determination of Fluoroquinolone Residues in Animal Tissues Using Escherichia coli as Indicator Organism. *Journal of AOAC International,* **82**(6):1407–1412.

Choma, I. M. (2000). Determination of four tetracyclines in milk by a simple Thin-Layer Chromatographic method. *Planar chromatography-Mod. TLC*, **13**:261*,* 159-169.

Code of Federal Regulations (1994). U.S. department of Health and Human Serv ices, Food and Drug Administration. (1 April) 21 CFR1, part 556.

Codex Alimentarius Commission (1993). List of Codex MRLs for vet drugs. *Res. Vet. drugs in foods,* **3**(1), FAO/WIK Rome.

Cunha, B. A. (2001). Antibiotic side effects. *Med. Clin. North. Am*., **85**(1):149-185.

Cupic, V., S. Dobric, D. Trailovic and Z. Pejcic (2004), Antimicrobial drugs in veterinary medicine, *Veterinarski glasnik*, **58**(5-6):577-94.

De Wasch, K., H. F. De Brabender, L. A. Van Ginkel, A. Spaan, S. S. Sterk and H. D. Meiring (1998). Confirmation of residues of thyreostatic drugs in thyroid glands by multiple mass spectrometry after thin-layer chromatography. *Journal of Chromatography A,* **819**:99-111.

Degroodt, J. M., B. W. Bukanski, J. De Groof and H. Beernaert (1991). Cimaterol and clenbuterol residue analysis by HPLC-HPTLC. *Z. Lbensmittel Untersuchung und Forschung,* **192**:430-432.

Di Corcia, A. and M. Nazzari (2002). Liquid chromatographic–mass spectrometric methods for analyzing antibiotic and antibacterial agents in animal food products. *Journal of Chromatography A*, **974:**53–89.

Dibner, J. J. and J. D. Richards (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poult. Sci*., **84**:634–643.

Dipeolu, M. A. and D. O. Alonge (2002). Residue of streptomycin in meat sold for human consumption in some states of SW Nigeria. *Arch. Zootec*., **51**:477-480.

Dipeolu, M. A. and D. O. Alonge (2002). Residues of streptomycin antibiotic in meat sold for human consumption in some states of south-western Nigeria. *Arch. Zootec.* **51**:477-480.

Donoghue, D. J. (2003). Antibiotic Residues In Poultry Tissues and Eggs: Human Health Concerns?. *Poultry Sci*., **82**:618–621.

Ellerbroek, L. (1991). New microbiological method for detection of Enrofloxacin, Ciprofloxacin and Flumequin, *Fleischwirtsch*., **71**:187-189.

Ellin Doyle M. (2001). Alternative to antibiotic use for growth promotion in animal Husbandary. FRI briefing. http://fri.wisc.edu/docs/pdf/antibiot.pdf.

Feinman, S. E. (1979). The Transmission of Antibiotic- Resistant Bacteria to People and Animals, *CRC Handbook Series in Zoonoses: Antibiotics, Sulfonamides, and Public Health*. CRC Press, Boca Raton, FL., pp. 71-151.

Fey, P. D., T. J. Safranek, M. E. Rupp, E. F. Dunne, E. Ribot, P. C. Iwen, P. A. Bradford, F. J. Angulo and S.H. Hinrichs (2000). Ceftriaxone-Resistant *Salmonella* Infection Acquired by a Child from Cattle, *The New England Journal of Medicine,* **342**(17):1242-1249.

Garcia, H. O., N. Gorla, A.Weyers, L. Ugnia, A. Magnoli (2004). Simultaneous quantification of ciprofloxacin, enrofloxacin and balofloxacin in broiler chicken muscle. *Arch. Med. Vet.*, 36(1):61-65.

Gashe, B. A. and S. Mpuchane (2000). Prevalence of *Salmonellae* on Beef Products at Butcheries and Their Antibiotic Resistance Profiles. *Journal of Food Science,* **65**(5):880-83.

Gaugain, M. and J. P. Abjean (1996). High-performance thin-layer chromatographic method for the fluorescence detection of three nitroimidazole residues in pork and poultry tissue. *Journal of Chromatography A,* **737:**343-346.

Gomes, E. R. and P. Demoly (2005). Epidemiology of hypersensitivity drug reactions. *Curr Opin Allerg Clin Immunol.,* **5**:309-316.

Gorla, N., E. Chiostri, L. Ugnie, A. Wereys, N. Giacomelli, R. Davicino and G. Ovando (1997). HPLC residues of enrofloxacin and ciprofloxacin in eggs of laying hens. *Int. J. Antimicrob. Agents*, **8**:253-256.

Guzman, A., C. Garcia, A. P. Marin, C. Willoughby and I. Demestre (2003). Developmental toxicity studies of the quinolone antibacterial agent irloxacin in rats and rabbits. *Arzneimittelforschung*, **53**:121-125.

Haasnoot, W., P. Stouten, G. Cazemier, A. Lommen, F. M. Nouws and H. J. Keukens (1999). Immunochemical detection of aminoglycosides in milk and kidney. *Anal.,* **124**:301-305.

Hassani, M., R. Lázaro, C. Pérez, S. Condón and R. Pagán (2008). Thermostability of oxytetracycline, tetracycline, and doxycycline at ultrahigh temperatures. J Agric Food Chem. **56**(8):2676-80.

Holmberg, S. D., J. G. Wells and M. L. Cohen (1984). Animal-to-Man Transmission of Antimicrobial-Resistant *Salmonella*: Investigations of U.S. Outbreaks, 1971-1983, *Science,* **225**:833-35.

Hussein, K. (2004). Experimental design for the microbiological four-plate test for the detection of sulphadimidine residues at the levels of concern. *Bull. Vet. Inst.* Pulawy, **48**(4):403-407.

Ibrahim, A. and W. A. Moats (1994). Effect of cooking procedure on oxytetracycline residue in lamb muscle. *Journal of agric. Food chem.,* **42:**2561-2563.

Ibrahim, A. I., A. U. Junaidu and M. K. Garba (2010). Multiple antibiotic residues in meat from slaughtered cattle in Nigeria. *The Internet Journal of Veterinary Medicine*, **8**(1).

Javadi A., H. Mirzaie and S. A. Khatibi (2011). Effect of roasting, boiling and microwaving cooking methods on Enrofloxacin residues in edible tissues of broiler. *African Journal of Pharmacy and Pharmacology,* **5**(2):214-218.

JECFA (1990). Evaluation of certain veterinary drug residues in food. WHO Technical Report Series, **799**:37-44.

Joshi, S. (2002). HPLC separation of antibiotics present in formulated and unformulated samples. *Journal of Pharmaceutical and Biomedical Analysis*, **28**:795–809.

Kabir, J., V. J. Umoh, , E. Audu-okoh, J. U. Umoh, and J. K. P. Kwaga (2004). Veterinary drug use in poultry farms and determination of antimicrobial drug residues in commercial eggs and slaughtered chicken in Kaduna State, Nigeria. *Food Control,* **15**:99-105.

Karraouan, B., B. Bouchrif, N. Ziyate, A. Talmi, K. I. S. Yahia, N. Cohen and A. Fassouane (2009). Evaluation of Multi-Plate Microbial Assay for the Screening of Antibacterial Residues in Poultry Muscle. *European Journal of Scientific Research,* **35**(2):311-317.

Kirbis, A. (2006). Microbilogical 5-plate screening method for detection of tetracyclines, aminoglycosides, cephalosporines and macrolides in milk. *Slo. Vet. Res*., **43**(4):161-168.

Kirubaharan, J. J., K. S. Palaniswami, K. Anubukumar and B. Mohanasubramaniam (1999). *In-virto* studies on antibacterial effect of crude garlic extract on *E.coli. Indian Veterinary Journal,* **76:**797-799.

Kozarova, I., J. Jasonova, D. Mate and S. Tkacikova (2009). Evaluation of three different microbial inhibition tests for the detection of sulphamethazine residues in the edible tissues of rabbit. *Food Addit. Contam. A.,* **26**:978-987.

Law, W. A. and L. N. Payne (1996). The poultry industry in poultry diseases, edited by F.T.W. Joordan and M. Pattison. 4th ed. W.B.Saunders company limited. London.

Lee, M. H., H. L. Lee and P. D. Ryu (2001). Public Health risks: Chemical and Antibiotic residues. *Asian-Australian J. Ani. Scien* **14**(3): 402-413.

Lemus, J. A., G. Blanco, J. Grande, B. Arroyo and M. Garcia-Montijano (2008). Antibiotics Threaten Wildlife: Circulating Quinolone Residues and Disease in Avian Scavengers. *PLoS ONE,* **3**(1):e1444.

Lolo, M., S. Pedreira, J. M. Miranda, B. I. Vazquez, C. M. Franco, A. Cepeda and C. Fente (2006). Effect of cooking on Enrofloxacin residues in chicken tissue*. F. Add. Cont. Part A*., **23**(10):988-993.

Luo, W. and C. Y. W. Ang (2000). Determination of Amoxicillin Residues in Animal Tissues by Solid-Phase Extraction and Liquid Chromatography with Fluorescence Detection. *Journal of AOAC International*, **83**(1):20-25.

Maraschiello C., E. Cusido, M. Abellan and J. Vilageliu (2001). Validation of an analytical procedure for the determination of the fluoroquinolone Enrofloxacin in chicken tissues. *J. Chromatogr. B: Biomed. Sci. Appl.,* **754** (2):311-318.

Martindale (2005). In: Sweetman, S., editor, The Complete Drug Reference, 34th ed., Pharmaceutical Press, London.

Martinez, M., P. McDermott and R. Walker (2005). Pharmacology of the fluoroquinolones: a perspective for the use in domestic animals, *The Vet Journal*, in press.

McEwen, S. A. and P. J. Fedorka-Cray (2002). Antimicrobial use and resistance in animals. *Clin Infect Dis*., **34**(3):S93-S106.

Mitchell, J. M., M. W. Griffiths, S. A. McEwen, W. B. McNab and A. J. Yee (1998). Antimicrobial Drug Residues in Milk and Meat: Causes, Concerns, Prevalence, Regulations, Tests, and Test Performance. *J Food Prot*., **61**:742–756.

Molbak, K., D. L. Baggesen, F. M. Aarestrup, J. M. Ebbesen, J. Engberg, K. Frydendahl, P. Gerner-Smidt, A. M. Petersen and H. C. Wegener (1999). An Outbreak of Multidrug-Resistant, Quinolone-Resistant *Salmonella enterica* Serotype Typhimurium DT104, *The New England Journal of Medicine,* **341**(19):1420-1425.

Musser, J. M. B., K. L. Anderson, J. E. Rushing and W. A. Moats (2001) Potential for Milk Containing Penicillin G or Amoxicillin to Cause Residues in Calves. *J. Dairy Sci.* **84**:126-133.

Myllyniemi, A. L., L. Nuotio, E. Lindfors, R. Rannikko, A. Niemi and C. Backman (2001). A microbiological six-plate method for identification of certain antibiotic groups in incurred kidney and muscle samples. *Anal.,* **126**:641-646.

Naeem, M., K. Khan and S. Rafiq (2006). Determination of residues of quinolones in poultry products by High Performance Liquid Chromatography. *Journal of applied sciences,* **6**(2):373-379.

National Academy of Sciences (1999). The use of drugs in food animals, benefits and risks. in Committee on Drug Use in Food Animals. Washington, D.C., National Academy Press.

Neidert, E., P. W. Saschenbrecker and F. Tittiger (1987). Thin layer chromatographic/ bioautographic method for identification of antibiotic residues in animal tissues. *J Assoc Off Anal Chem*., **70**(2):197-200.

Niewold, T. A. (2007). The non antibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poult. Sci*., **86**:605–609.

Nisha, A.R. (2008). Antibiotic Residues - A Global Health Hazard. *Veterinary World*, **1**(12): 375-377.

Nonga, H. E., M. Mariki, E. D. Karimuribo and R. H. Mdegela (2009). Assessment of Antimicrobial Usage and Antimicrobial Residues in Broiler Chickens in Morogoro Municipality, Tanzania. *Pakistan Journal of Nutrition*, **8**(3):203-207.

O'brien, J. J., N. Campbell and T. Conaghan (1981). Effect of cooking and cold storage on biologically active antibiotic residues in meat. *J. Hyg., Camb.* **87**:511-523.

Oka, H., Y. Ikai, N. Kawamura, K. Uno, M. Yamada and M. Suzuki (1987). Improvement of chemical analysis of antibiotics XII. Simultaneous analysis of seven tetracyclines in honey. *J. Chromatogr*., **400:**253-261.

Oka, H., Y. Ito and H Matsumoto (2000). Chromatographic analysis of tetracycline antibiotics in foods. *Journal of Chromatography A*, **882**:109–133.

Okerman, L., H. Noppe, V. Cornet and L. De Zutter (2007). Microbiological detection of 10 quinolone antibiotic residues and its application to artificially contaminated poultry samples. *Food Addit Contam.,* **24**:252–257.

Okerman, L., J. Van Hoof, W. Debeuckelaere (1998). Evaluation of the European four-plate test as a tool for screening antibiotic residues in meat samples from retail outlets. *J. Assoc. off. Anal. Chem*., **81**:51-56.

Paturkar, A. M., V. S. Waskar, K. V. Mokal and R. J. Zende (2005). Antimicrobial drug residues in meat and their public health significance - a review. *Ind J Anim Sci*., **75**:1103-1111.

Pavlov, A., L. Lashev and V. Rusev (2005). Studies on the residue levels of tobramycin in stored poultry products. *Trakia J Sci.*, **3**(5):20-22.

Petrovic, J., M. Baltic, V. Cupic, S. Stefanovic and D. Stojanovic (2006). Residues of enrofloxacin and its main metabolite ciprofloxacin in broiler chickens. *Acta Veterinaria (Beograd)*, **56**(5-6):497-506.

Popelka, P., J. Nagy, S. Marcincak, P. Jevinova and K. Hussein (2003). Comparison of bsda and premi® test sensitivity to penicillin standards in poultry meat and after administration of amuril. *Folia Veterinaria.*, **7**(3):73-81.

Raison-Peyron, N., D. Messaad, J. Bousquet and P. Demoly (2001). Anaphylaxis to beef in penicillin-allergic patient. *Allergy,* **56**:796-797.

Ramrrez, A., R. Gutierrez, G. Draz, C. Gonzalez, N. Perez and S. Vega (2003). High-performance thin-layer chromatography-bioautography for multiple antibiotic residues in cow's milk. *Journal of Chromatography B*, **784:**315–322.

Roca, M., M. Castillo, P. Marti, R. L. Althaus and M. P. Molina (2010). Effect of Heating on the Stability of Quinolones in Milk. *Journal of Agricultural and Food Chemistry*, **58**(9):5427-5431.

Rose, M. D., G. Shearer and W. H. H. Farrington (1995). The effect of cooking on veterinary drug residues in food: 3. Sulfamethazine. *Food Additives and Contaminants,* **12:**739–750.

Rose, M. D., J. Bygrave, W. H. H. Farrington and G. Shearer (1996). The effect of cooking on veterinary drug residues in food: 4. Oxytetracycline. Food Additives and Contaminants, **13**:275–286.

Salehzadeh, F., A. Salehzadeh, N. Rokni, R. Madani and F. Golchinefar (2007). Enrofloxacin residue in chicken tissues from Tehran slaughter houses in Iran. *Pak. J. Nutr*., **6**(4):409-413.

Salehzadeh, F., R. Madani, A. Salehzadeh, N. Rokni and F Golchinefar (2006). Oxytetracycline Residue in Chicken Tissues from Tehran Slaughterhouses in Iran. *Pakistan Journal of Nutrition,* **5**(4):377-381.

Sawant, A. A., L. M. Sordillo and B. M. Jayarao (2005). A Survey on Antibiotic Usage in Dairy Herds in Pennsylvania. *Journal of Dairy Science*, **88**(8):2991-2999.

Shahid, M. A., M. Siddique, S. U. Rehman, S. Hameed and A. Hussain (2007). Evaluation of a Microbiological Growth Inhibition Assay as a Screening Test for the Presence of Antibiotic Residues in Poultry Meat. *American journal of food technology*, **2**(5):457-461.

Shareef, A. M., Z. T. Jamel and K. M. Yonis (2009). Detection of antibiotic residues in stored poultry products. *Iraqi Journal of Veterinary Sciences*, **23**(1):45-48.

Simonsen, G. S., H. Haaheim, K. H. Dahl, H. Kruse, A. Lovseth, O. Olsvik and A. Sundsfjord (1998). Transmission of Van A-type vancomycin resistant enterococci and Van A resistance elements between chicken and humans at avoparcin-exposed farms. *Microb Drug Resist Winter.*, **4**(4):313 -318.

Singh, S. (2010). Determination of antibiotic residues in milk samples of dairy animal in jabalpur. MVSc, Thesis submitted to J.N.K.V.V., Jabalpur.

Skoog, D. A., F. J. Holler and T. A. Nieman (1998). Principles of Instrumental Analysis. 5th ed. *Saunders, Philadelphia*. USA.

Smith, J. L., D. J. Drum, Y. Dai, J. M. Kim, S. Sanchez, J. J. Maurer, C. L. Hofacre and M. D. Lee (2007). Impact of antimicrobial usage on antimicrobial resistance in commensal *Escherichia coli* strains colonizing broiler chickens. *Appl Environ Microbiol.*, **73**(5):1404-14.

Smither, R., A. F. Lott, R. W. Dalziel and D. C. Ostler (1980). Antibiotic residues in meat in the United Kingdom; an assessment of specific tests to detect and identify antibiotic residues. *J. Hyg., Camb*., **85**:359-369.

Stolker, A. A. and U. A. Brinkman (2005). Analytical strategies for residue analysis of veterinary drugs and growth promoting agents in food-producing animals-a review. *Journal of chromatography A.,* **1067**:15-53.

Tajick, M. A. and B. Shohreh (2006). Detection of Antibiotics Residue in Chicken Meat Using TLC. *International J Poultry Sci*., **5** (7):611-612.

Tajick, M. A., H. Rahimian, A. Alizadeh and V. Rezaeian (2002). Analysis of isolated lipids from sclerotia of *Rhizoctonia solani* AG-1-1A using TLC. Proceeding of 14th Iranian Plant Protection Congress, Kermanshah, Iran, P: 291.

Telling, G. M. (1990). Control of Veterinary drug residues in Food. *Proceedings of the Euro-Residue Conference,* Noordwij-kerhout, The Netherlands, 21-23 May.

Thangadu, S., S. K. Shukla and Y. Anjaneyulu (2002). Separation and detection of certain β - lactams and floroquinolone antibiotic drugs by thin layer chromatography. *Analytical Sci*., **18**:97-100.

Traub, W. H. and B. Leonhard (1995). Heat stability of the antimicrobial activity of sixty-two antimicrobial agents. Journal of antimicrobial chemotherapy, **35**:149-154.

U.S. Department of Agriculture, Food Safety and Inspection Service (1997). *Salmonella Typhimurium DT104 Situation Assessment*. Dec. 1997.

Van Egmond, H. J., J. F. M. Nouws, R. Schilt, W. D. M. Van Lankveld-Driessen, N. E. P. M. Streutjens-van and F. G. H. Simons (2000). Stability of antibiotics in meat during a stimulated high temperature destruction process. Proceedings of The Euro Residue conference IV, Veldhoven, Netherlands, pp. 430-438.

Van Poucke, L. S. G., G. C. I. Depourcq and C. H. Van Peteghem (1991). A quantitative method for the detection of sulphonamide residues in meat and milk samples with a high-performance thin-layer chromatographic method. *Journal of Chromatography,* **29:**423-427.

Vollard, E. J. and H. A. L. Clasener (1994). Colonization resistance. *Antimicrobial Agents Chemotherapy,* **38**:409–414.

Waltner-Toews, D. and S. A. McEwen (1994). Residues of antibacterial and antiparasitic drugs in foods of animal origin - a risk assessment. *Prev Vet Med*., **20**:219-234.

White, D. G., S. Zhao, R. Sudler, S. Ayers, S. Friedman, S. Chen, P. F. McDermott, S. McDermott, D. D. Wagner and J. Meng (2001). The Isolation of Antibiotic-Resistant *Salmonella* from Retail Ground Meats, *The New England Journal of Medicine,* **345**(16):1147-54.

Whitehead, H. R. and G. A. Cox (1956). The detection of penicillin in milk. *J. Appl. Bact*., **19**(2):247-249.

WHO (1997). Division of Emerging and other Communicable Diseases Surveillance and Control. The Medical impact of the use of antimicrobials in food animals : report of a WHO meeting, Berlin, Germany, 13-17 October 1997., Geneva: World Health Organization. 24 p.

WHO (2003). Dept. of Communicable Disease Prevention Control and Eradication, Danish Veterinary Institute., and Danmarks jordbrugsforskning, Impacts of antimicrobial growth promoter termination in Denmark : the WHO international review panel' s evaluation. edited by M. Barza and S. L. Gorbach. The termination of the use of antimicrobial growth promoters in Denmark : Foulum, Denmark 6-9 November 2002., Geneva: World Health Organization. p.57

Wibawa, J. I. D., D. Fowkes, P. N. Shaw and D. A. Barrett (2002). Measurement of amoxicillin in plasma and gastric samples using highperformance liquid chromatography with fluorimetric detection. *J Chromatogr B.,* **774**:141-148.

Wolin, A. G. and F. V. Kosikowski (1958). Formation of bacterial inhibitory zones in whey agar by raw milk. *J. Dairy Sci*., **41**:34-40.

Woodward, K. N. (2004). The toxicity of particular veterinary drug residues, pp. 175-223. In Pesticide, veterinary and other residues in food. Watson DH (ed.), Woodhead Publishing Ltd., New York.

World Health Organization (2009). Division of Emerging and other Communicable Diseases

Xu, W., X. Zhu, X. Wang, L. Deng and G. Zhang (2006). Residues of Enrofloxacin, furazolidone and their metabolites in Nile tilapia (*Oreochromis niloticus*). *Aqua*., **254**:1–8.

Yonova, I. (1971). Studies on thermal resistance of tetracycline and oxytetracycline residues in eggs and poultry meat. *Veterinarnomed. Nauki.,* **8**(10):75-82.

Zorraquino, M. A., M. Roca, N. Fernandez, M. P. Molina and R. Althaus (2008). Heat Inactivation of ß-lactam Antibiotics in Milk. *Journal of Food Protection,* **71**(6):1193–1198.

Zucker, A.D. and M. Levy (2008). The relationship between antimicrobial consumption and Human Emerging Infectious Disease. Consortium for Conservation Medicine.

VITA

The author Dr. Mahesh S. Trivedi was born on 2 April 1985 at Mugawali, M.P.. He completed his intermediate in the year 2002 with 66% and took admission in the college of Veterinary Science and Animal Husbandry, Jabalpur in the year 2005, and completed his graduation with an OGPA of 8.22 on 10 point scale. Being interested in the field of Pharmacology and Toxicology, he took admission for postgraduation in the discipline in the same college and completed MVSc degree with an OGPA of 8.26 on 10 point scale.